THE TAXONOMY AND SIGNIFICANCE OF *Chryseobacterium* ISOLATES FROM POULTRY

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THE TAXONOMY AND SIGNIFICANCE OF *Chryseobacterium* ISOLATES FROM POULTRY

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DECLARATION

I George Charimba, declare that the thesis hereby submitted by me for the Ph.D. degree in the Faculty of Natural and Agricultural Sciences 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 this thesis in favour of the University of the Free State.

G. Charimba
January, 2012

To my wife Eunice, my children Millicent, Tariro and George Jr.

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(This thesis was written according to the typographical style of the International Journal of Systematic and Evolutionary Microbiology)

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of three determinations. (■) *C. carnipullorum* 9_R23581^{T}; (♦) *C. shigense* DSM 17126^{T}; (●) *C. gleum* NCTC 11432^{T}; (▲) *Streptomyces* sp. DSM 40758; (×) *B. cereus* ATCC 10876^{TM}

LIST OF ABBREVIATIONS

AP-PCR	Arbitrarily primed PCR
AFLP	Amplified fragment length polymorphism
ARDRA	Amplified rDNA restriction analysis
ATCC	American Type Culture Collection, Manassas, Virginia
A_w	Water activity
BPW	Buffered peptone water
cfu	Colony forming units
C.	*Chryseobacterium*
^oC	Degrees Celcius
DNA	Deoxyribonucleic acid
DDH	DNA-DNA hybridization
E	*Elizabethkingia*
Ed(s)	Editor(s)
eg	For example
et al.	(*et alii*) and others
F.	*Flavobacterium*
FAME	Fatty acid methyl esters
Fig.	Figure
g	gram
G+C	Guanine plus cytosine
GN	Gram negative
h	Hour
kg	Kilogram
LMG	Laboratory for Microbiology, University of Ghent
LMW	Low molecular weight
log	Log_{10}
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
Mol	Mole

Mol%	Mole percent
NA	Nutrient Agar
NCTC	National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom
ND	Not detected
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PM	Phenotype MicroArray
pp	Page(s)
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal Ribonucleic Acid
rep-PCR	Repetitive element sequence-based PCR
sec	second(s)
sp.	Species or unknown/unidentified/unspecified species
SPCA	Standard plate count agar
TBC	Total bacteria count
TYCC	Total yellow colonies count
T_m	melting temperature
™	Trade mark
UK	United Kingdom
USA	United States of America
µl	Microlitre
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background to the study

Members of the genus *Chryseobacterium,* in reflection of its family *Flavobacteriaceae,* are ubiquitous in nature and are common contaminants of meat and poultry (Bernardet *et al*., 2006). The taxonomy of these yellow-pigmented, Gram-negative rods was rooted in the taxonomy of the erstwhile genus *Flavobacterium*.

A few decades back, most studies described bacteria based on certain phenotypic traits such as shape, colony colour, cell size, staining properties, motility, host range, pathogenicity, and assimilation of a few carbon sources (Prakash *et al*., 2007). Since the 1970's, polyphasic taxonomy has gained in prominence integrating genotypic, chemotypic and phenotypic characteristics in order to classify organisms into their natural groups. Advances in 16S rRNA gene sequence analysis and molecular fingerprinting techniques revolutionized prokaryote systematics. The new techniques together with the traditional ones are key elements in determining whether unknown strains belong to known taxa or whether they constitute novel ones (Tindall *et al*., 2010). Consequently, the taxonomy of bacteria went through many changes and the flavobacteria were no exception.

In 1923, the genus *Flavobacterium* consisted of 46 yellow-pigmented mainly Gram-negative, rod-shaped, non-endospore forming, chemoorganotrophic bacteria. It was far from homogeneous since all yellow-pigmented poorly described taxa were placed in this genus (Weeks, 1981). The history of the genus is a record of proposals attempting to achieve credibility for this taxonomic group (Holmes, 1992). One of the milestones of this history was the suggestion by Jooste (1985) for the genus *Flavobacterium* to be accommodated in a new family, the *Flavobacteriaceae*, with three genera (Holmes, 1992). This was accepted by

Reichenbach in 1989 and validated in 1992 (Reichenbach, 1992). It provided the genus *Flavobacterium* with an affiliation. To date the family has 94 genera (Euzéby, 2012a).

The genus *Chryseobacterium* was proposed by Vandamme *et al*. (1994) to accommodate six renamed and regrouped flavobacterial strains following the demise of the erstwhile genus *Flavobacterium*. The renamed species were *Chryseobacterium* [*F*.] *indologenes*, *C*. [*F*.] *gleum*, *C*. [*F*.] *indoltheticum*, *C*. [*F*.] *balustinum* and *C.* [*F*.] *meningosepticum* (Bernardet *et al.,* 2006). The fish pathogen, *C*. [*F*.] *scopthalmum* (Mudarris *et al.,* 1994) was also included in this genus since it belonged to the same rRNA cluster. In 2005, two species were relocated to the new genus *Elizabethkingia* (Kim *et al*., 2005). Two *Sejongia* species were transferred to the genus *Chryseobacterium* (Kämpfer *et al*., 2010b). Furthermore, species of the genus *Kaistella* were also transferred to the genus *Chryseobacterium* (Kämpfer *et al.,* 2009b). Kämpfer *et al*. (2010) proposed the reclassification of *Chryseobacterium arothri* (Campbell *et al*., 2008) as a later heterotypic synonym of *Chryseobacterium hominis* (Vaneechoutte *et al*., 2007). Meanwhile the number of new species continued to grow rapidly from 11 species in 2005 to 58 to date and more continue to be described (Euzéby, 2012b).

The genus *Chryseobacterium* has long been associated with food spoilage and proteolytic activity (Jooste *et al*., 1986; Vandamme *et al*., 1994; Forsythe, 2000). Its broader role in the food and feather industry has not been studied in as much detail as its taxonomy. The poultry industry produces huge amounts of feather waste and it causes disposal problems. Feathers are constituted of almost pure keratin protein, which is insoluble and undegradable by most proteolytic enzymes. Some *Chryseobacterium* species have been shown to produce keratinolytic enzymes that degrade chicken feathers and possess potential in biotechnological, non-polluting processes involving keratin hydrolysis (Riffel *et al*., 2007).

The applications of members of the family *Flavobacteriaceae* are related to their habitats and their relationship to the hosts. Beneficial aspects include synthesis of a number of potentially useful enzymes in industry or medicine; turnover of

organic matter in soil, water and sewage plants; decomposition of pesticides and insecticides; destruction of toxic proliferative algae; and symbiosis with various insects (Bernardet and Nakagawa, 2006). The negative aspects include spoilage and defects of food such as poultry, meat, fish, milk and dairy products; infections in humans and animals; and destruction of valuable algae and vegetables (Holmes, 1984a; Bernardet and Nakagawa, 2006).

There is little information on keratinases produced by Gram-negative bacteria. Recently, however, *Chryseobacterium* strain kr6, was reported to produce keratinases which degraded chicken feathers (Brandelli and Riffel, 2005).

1.2 Purpose, hypotheses and objectives of the study

1.2.1 *Purpose*

i) To carry out further research on members of the family *Flavobacteriaceae*, with special reference to the genus *Chryseobacterium* in order to obtain more knowledge and better understanding of their characteristics and correct taxonomic status. This was mainly motivated by its changing taxonomy and the proven and potential significance as well as possible applications of its members.

ii) To subject a range of recently and previously isolated chryseobacteria obtained from poultry sources to the latest taxonomic techniques to more accurately characterize and classify them.

iii) To describe and name any new species that might emerge from the comprehensively characterized strains.

iv) To explore a possible application of the novel species in the degradation of the recalcitrant poultry feathers.

1.2.2 *Hypotheses*

i) *Chryseobacterium* species will occur on poultry feathers since they have been isolated from raw chicken.

ii) The examination and re-examination of recently and previously isolated *Chryseobacterium* strains obtained from poultry sources using newer phenotypic and molecular techniques will reveal their exact taxonomic identities.

iii) Phenotype MicroArray substrate utilization by representative strains will give an indication of broader potential applications.

iv) Screening of poultry sources for *Chryseobacterium* strains will yield keratinolytic strains that are able to degrade feathers since enzymes which are produced by these organisms are induced in response to the most abundant source of nutrition.

Hypothesis i) will be tested in Chapter 3; hypothesis ii) will be tested in Chapters 4 and 5; hypothesis iii) will be tested in Chapter 6 and hypothesis iv) will be tested in Chapter 7.

1.2.3 *Objectives*

i) To isolate *Chryseobacterium* strains from chicken feather waste and perform preliminary characterization using the BIOLOG Gen II identification system.

ii) To classify the raw chicken and feather waste isolates using 16S rRNA gene sequence analysis, conventional phenotypic tests and the BIOLOG Omnilog Gen III identification system.

iii) To perform a polyphasic taxonomic study using DNA-DNA hybridization, chemotaxonomic and biochemical tests, and describe and name possible new species.

iv) To perform a Phenotype MicroArray characterization of selected strains using the BIOLOG Omnilog PM system.

v) To investigate the keratinolytic activity of the novel species using chicken feathers as the source of carbon, nitrogen, sulphur and phosphorus.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The genus *Chryseobacterium* belongs to the family *Flavobacteriaceae* (Bernardet *et al*., 2011). It was first mooted by Vandamme *et al.* (1994a) based on rRNA studies. At that juncture, it consisted of six species (*Chryseobacterium balustinum*, *C. gleum* [the type species], *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum*) that were relocated from the erstwhile genus *Flavobacterium*.

The family *Flavobacteriaceae* was first suggested by Jooste (1985) in his Ph.D. study and was then mentioned by Reichenbach (1989) and placed in the order *Cytophagales* in the first edition of *Bergey's Manual of Systematic Bacteriology* even though it was not formally described (Holmes, 1997; Bernardet *et al.,* 2006). The family was later validated and had its description published (Bernardet *et al.,* 1996). The family emerged as yellow-orange to non-pigmented, non-gliding, strictly aerobic organisms retrieved from a variety of environments and from clinical specimens (Bernardet, 2011). However, considerable modifications occurred to the description and taxonomy of the family since its publication with the objective to achieve homogeneity and a valid taxonomic status (Bernardet *et al.,* 1996; Bernardet *et al.,* 2002, Bernardet and Nakagawa, 2006; Bernardet, 2011). Following the demise of the erstwhile genus *Flavobacterium*, its ruins gave way to the birth of several new genera into which were placed many of the species that were associated with food spoilage and pathogenicity. These new genera included *Bergeyella*, *Chryseobacterium, Empedobacter, Myroides, Weeksella and Flavobacterium* (type genus; Holmes 1992; Hugo and Jooste, 2003; Bernardet and Nakagawa, 2006).

The *Chryseobacterium* genus includes food spoilage microorganisms that are ubiquitous and occur in a variety of ecological niches (Hugo *et al.,* 2003; 2012; de

Beer *et al.,* 2005a; de Beer *et al.,* 2006) but their significance in the food industry has long been debated. Poultry feathers for example, have been shown to harbour *Chryseobacterium* strains with very high keratinolytic activity (Casarin *et al.,* 2008). These strains were able to break down the insoluble keratin in feathers by the production of keratinases (Riffel *et al.,* 2003; Casarin *et al.,* 2008). Relatively heat stable keratinases which degrade feathers are important in potential industrial processes that break down poultry feathers yielding digestible or accessible proteins and amino acids.

The aims of this literature review will, therefore, be to elucidate on the evolution of the taxonomy of the family *Flavobacteriaceae* with special reference to the genus *Chryseobacterium*, sources of isolation and description. Secondly, techniques for polyphasic taxonomic studies applicable to the *Flavobacteriaceae* family will be discussed. The third aim will be to illustrate the significance of chryseobacteria in clinical, food and industrial sources, with special emphasis on the two last mentioned sources. Finally, the role of *Chryseobacterium* strains in keratinolysis and its applications will also be discussed.

2.2. Taxonomy of the *Flavobacteriaceae* family

2.2.1. *Historical Overview*

The family *Flavobacteriaceae* had its inception in the genus *Flavobacterium*. In the first edition of *Bergey's Manual of Determinative Bacteriology*, Bergey *et al.* (1923) proposed the name *Flavobacterium* for a genus of the family *Bacteriaceae*. It consisted of 46 yellow-pigmented mainly Gram-negative, rod shaped, non-endospore forming, chemoorganotrophic bacteria. The genus was placed in the tribe *Chromobacteridales*. The tribe had three other genera of aerobic bacteria which were separated from each other by the production of differently coloured pigments namely *Chromobacterium* (purple pigment), *Pseudomonas* (green fluorescent pigments) and *Serratia* (red pigments; Holmes, 1992). Subsequently, all yellow-pigmented poorly described taxa were placed in the genus *Flavobacterium* (Weeks, 1981). Polar flagellates were removed from the genus in the fifth edition of *Bergey's Manual of Determinative Bacteriology*

(Bergey *et al.,* 1939). In the sixth edition, the genus was grouped together with *Alcaligenes* and *Achromobacter* in the family *Achromobacteriaceae* (Bergey and Breed, 1948). Gram-positive species were removed from the genus in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Weeks and Breed, 1957) leaving 26 species. In the eighth edition, 14 species were removed leaving 12 species that were grouped in two groups of six species each based on the mole percent guanine + cytosine content (mol% G+C). Group I consisted of low mol% G+C content of 26% to 40%, while group II had a high mol% G+C content of 63% to 70% (Weeks, 1974). The group II organisms were removed in the 1984 edition. At that time, members of the genus *Flavobacterium* were described as Gram-negative, yellow, non-motile, aerobic rods usually growing at 5–30 ^{o}C, and isolated from environmental and clinical sources (Holmes *et al.,* 1984a).

In 1985, Jooste proposed the family *Flavobacteriaceae* which was accepted by Reichenbach (1989) in the first edition of *Bergey's Manual of Systematic Bacteriology* even though it was not formally described (Holmes, 1997; Bernardet *et al.,* 2002). The family was later validated by citation on a validation list (Reichenbach, 1992) and an emended description was later published (Bernardet *et al*., 1996). It consisted of eight genera; *Flavobacterium*, *Bergeyella*, *Capnocytophaga*, *Chryseobacterium*, *Empedobacter*, *Ornithobacterium*, *Riemerella*, and *Weeksella* and the organisms that would later become *Myroides* and *Tenacibaculum*. The family description was based on the features of the genus *Flavobacterium* which was then described as yellow-orange to non-pigmented, non-gliding, strictly aerobic organisms retrieved from various environmental sources and clinical specimens that may become pathogenic (Bernardet *et al.,* 2009).

In the early 1980's and 1990's, the taxonomy of the flavobacteria again underwent some changes. The genus *Flavobacterium* was restricted to non-motile and non-gliding species (Holmes *et al.*, 1984a). It was further restricted when it was recognised that the type species, *Flavobacterium aquatile*, did not represent the genus (Holmes, 1993). As a result, *Flavobacterium aquatile* was set aside in Holmes taxonomic review in the second edition of *The Prokaryotes*

(Holmes, 1992) and was only reinstated as the type species following a decision by the Judicial Commission of the International Committee on Systematic Bacteriology even though the genus had been thoroughly emended (Bernardet *et al.,* 1996).

In the second edition of *The Prokaryotes*, Holmes (1992) recognized four natural groups of flavobacteria species, namely A, B, C and D, based on habitat, resistance to antimicrobial agents, production of yellow pigment and indole, oxidation of carbohydrates and proteolytic activity. Table 2.1 shows the differentiation of flavobacteria according to Holmes (1992). Groups B, C, and D rapidly became the basis for the following genera respectively: *Myroides* (Vancanneyt *et al.*, 1996), *Sphingobacterium* (Yabuuchi *et al*., 1983) and *Weeksella* and *Bergeyella* (Holmes *et al.,* 1986a; 1986b, Vandamme *et al.,* 1994a).

Group A of Holmes (1992) comprised strains previously known as CDC Group IIa ([*Flavobacterium*] *balustinum*, [*F*.] *breve*, [*F*.] *indoltheticum*, [*F*.] *meningosepticum*) and CDC Group IIb ([*F*.] *indologenes* and [*F*.] *gleum*; King, 1959). Squared brackets indicate generically misclassified bacteria. The heterogeneity of these strains was long established and following phylogenetic studies by Vandamme *et al.* (1994a), the new genus *Chryseobacterium* was proposed for Group A. Former flavobacteria were regrouped in this genus and renamed on the basis of fitting into a tight rRNA cluster. The renamed species were *Chryseobacterium* [*F*.] *indologenes*, *C*. [*F*.] *gleum*, *C*. [*F*.] *indoltheticum*, *C*. [*F*.] *balustinum* and *C.* [*F.*] *meningosepticum* (Bernardet *et al.,* 2006). The then recently described fish pathogen, *C*. [*F*.] *scopthalmum* was also included in this genus since it belonged to the same rRNA cluster (Mudarris *et al.,* 1994). *Chryseobacterium gleum* was chosen as the type species over the genus' two oldest species, *C. balustinum* and *C. indoltheticum*, as well as the well characterized, clinically important but most aberrant member of the genus, [*C*]. *meningosepticum* because its description was based on firm genotypic and phenotypic grounds following an extensive comparative study of 12 strains (Holmes *et al.,* 1984a; Bernardet *et al.,* 2006). Vandamme *et al.* (1994a) also proposed to revive the name *Empedobacter* to accommodate [*F*.] *breve* which

occupied a separate position in the rRNA cluster and a new combination *Empedobacter brevis* was established.

A new genus, *Elizabethkingia*, was later proposed by Kim *et al.* (2005a) after a polyphasic study of several [*C*.] *meningosepiticum* strains and the only available [*C*.] *miricola* strain and the new combinations *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* were established. These recently described species occupied a separate position compared to all *Chryseobacterium* species as shown in Figures 2.1 and 2.4.

2.2.2. *Current Taxonomy*

The family *Flavobacteriaceae* belongs to the domain Bacteria, phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB; Hirsch *et al.,* 1998). Together with the families *Bacteroidaceae*, *Cytophagaceae*, *Sphingobacteriaceae*, *Spirosomaceae, Cryomorphaceae* and *Blattaebacteriaceae*, the family *Flavobacteriaceae* belongs to the class *Flavobacteriia*, phylum *Bacteroidetes*, domain Bacteria (Bernardet, 2011). The phylum *Bacteroidetes* was previously known by the names "*Cytophaga-Flavobacterium-Bacteroides* group (CFB)", "*Flavobacterium-Bacteroides* phylum" and rRNA superfamily V (Bernardet *et al.,* 2002).

2.2.3. *Phylogeny*

Results from extensive analysis of 16S rRNA/rDNA sequences and DNA-rRNA hybridization experiments performed over the past two decades, formed the basis of the more accurate and reliable current phylogenetic relationships (Bernardet and Nakagawa, 2006). Yamamoto and Harayama (1996) analysed the DNA gyrase B subunit gene (*gyr*B) and concluded that it may have a greater degree of resolution than the phylogenetic analysis based on 16S rRNA gene sequences because protein encoding genes evolve faster than rRNA genes. Phylogenetic studies were also deduced from signatures in different protein sequences.

Table 2.1. Differentiation of the flavobacteria (Holmes, 1992).[a]

Character	Habitat	Resistant to anti-microbial agents	Yellow pigment	Indole production	Oxidation of carbo-hydrates	Proteolytic activity
Group A: *F. balustinum, F. breve* *Flavobacterium* species Group IIb (*F. gleum*, *F. indologenes*) *F. indoltheticum* *F. meningosepticum*	Free-living	+	+	+	+	+
Group B: *F. odoratum*	Free-living	+	+	-	-	+
Group C: *F. mizutaii* *F. multivorum* *F. spiritivorum* *F. thalpophilum* *F. yabuuchiae*	Free-living	+	+	-	+	-
Group D: *Weeksella virosa* *W. zoohelcum*	Strict saprophyte	-	-	+	-	+

[a] +: positive reaction; -: negative reaction.

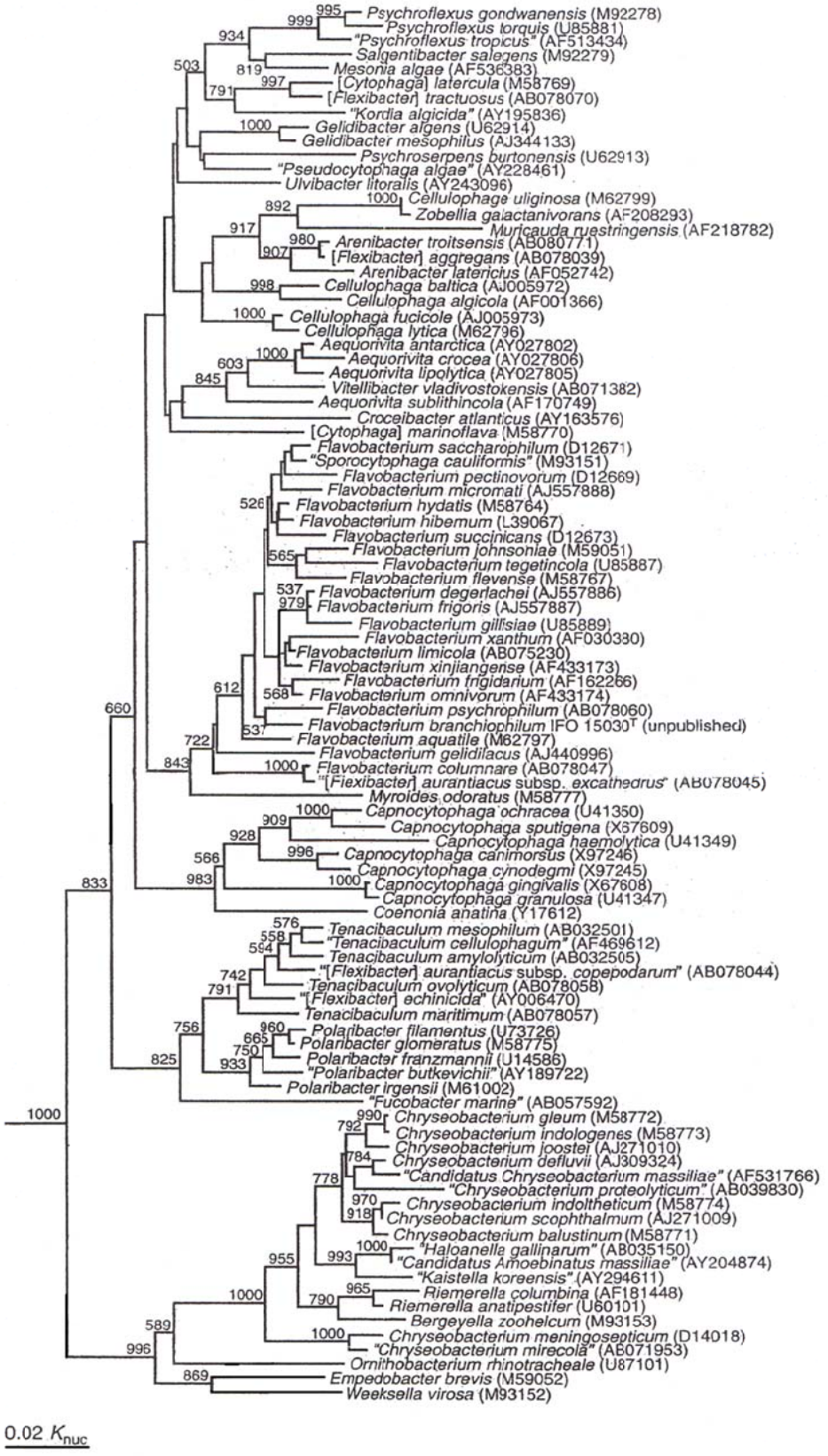

Fig. 2.1. Phylogenetic relationships among representatives of the family *Flavobacteriaceae* based on comparisons of 16S rRNA gene sequence. The number of nucleotides compared was 899 bp. *Agrobacterium tumefaciens*, *Bacillus subtilis* and *Escherichia coli* were used as outgroups (Bernardet and Nakagawa, 2006).

However, other publications revealed that despite the differences in their degree of resolution, various molecules used for phylogenetic analysis yield concordant data (Gupta, 2000; Suzuki *et al.,* 2001). Figure 2.1 outlines the phylogenetic relationships among members of the *Flavobacteriaceae* family based on 16S rRNA gene sequences. Differences in 16S rRNA gene sequences of up to 5% have been found among strains of some species included in the family (Clayton *et al.,* 1995; Triyanto and Wakabayashi, 1999), and hence there is need to obtain and compare sequences of additional strains when using this technique (Bernardet and Nakagawa, 2006). Analysis of 16S rRNA gene sequences should be restricted to the generic and suprageneric levels since this technique's resolution is not adapted to the delineation of new species (Bernardet *et al.,* 2002). However, procedures are now available for the determination of almost complete 16S rRNA gene sequences wherein resolution of phylogenetic relationships can be achieved much more accurately and reliably (Bernardet *et al.,* 2002).

Table 2.2 lists the genera in the family *Flavobacteriaceae* as well as their type species, and their sources of isolation. Although the genus *Fucobacter* is included in this table it is not in the *List of Prokaryotic Names with Standing in Nomenclature* since it was not published in the *International Journal of Systematic and Evolutionary Microbiology* and not included in a validation list in this journal. At present there are 94 flavobacteria genera in the *List of Prokaryotic Names with Standing in Nomenclature* (Euzéby, 2012).

2.2.4. *Description of the family Flavobacteriaceae*

The following emended description of the family *Flavobacteriaceae* Reichenbach 1989 is according to Bernardet *et al.* (2002) and is until now, the most recent:

Family I. *Flavobacteriaceae* Reichenbach 1992, 327^{vp} (Effective publication: Reichenbach 1989, 2013) emend. Bernardet, Segers, Vancanneyt, Berthe, Kersters, and Vandamme 1996, 145^{vp}

Cells are short to moderately long rods with parallel or slightly irregular sides and rounded or slightly tapered ends. They are usually 0.3-0.6 μm wide and 1-10 μm long, though members of some species may form filamentous flexible cells (e.g. *Flavobacterium* and *Tenacibaculum*) or coiled and helical cells (e.g. *Polaribacter*, *Psychroflexus* and *Psychroserpens* strains) under certain growth conditions; ring shaped cells are not formed. Cells in old cultures may form spherical or coccoid bodies (e.g. *Flavobacterium*, *Gelidibacter*, *Psychroserpens* and *Tenacibaculum*). Gram-negative. Non-spore forming. Gas vesicles are produced in some members of *Polaribacter* species. Flagellae are usually absent; the only *Polaribacter irgensii* strain available is flagellated, but motility has not been observed in wet mounts. Non-motile (*Bergeyella*, *Chryseobacterium*, *Coenonia*, *Empedobacter*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, and *Weeksella* strains, and *Psychroflexus gondwanensis* strains) or motile by gliding (*Capnocytophaga*, *Cellulophaga*, *Gelidibacter*, *Flavobacterium*, *Tenacibaculum* and *Zobellia* strains, and *Psychroflexus torquis* strains).

Growth is aerobic (*Bergeyella*, *Cellulophaga*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Weeksella* and *Zobellia* strains). The optimum temperature is usually in the range 25–35 ^{o}C, but members of some species or genera are psychrotrophic or psychrotolerant (*Flavobacterium psychrophilum*, and the Antarctic *Flavobacterium* species, as well as *Gelidibacter*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum* and *Zobellia* strains).

Colonies are non-pigmented (*Bergeyella*, *Coenonia*, *Ornithobacterium* and *Weeksella* strains) or pigmented by carotenoid or flexirubin pigments or both (*Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Empedobacter,* *Flavobacterium*, *Gelidibacter, Myroides, Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum* and *Zobellia* strains).

Menaquinone 6 is either the only respiratory quinone or the major respiratory quinone. Chemo-organotrophic. Intracellular granules of poly-β-hydroxybutyrate

are absent. Sphingophospholipids are absent. Homospermidine is the major polyamine though agmatine, cadaverine and putrescine are frequently present as minor components. Crystalline cellulose (i.e. filter paper) is not decomposed. The DNA base composition ranges from 27 to 44 mol% G+C.

Mostly saprophytic in terrestrial and aquatic habitats. Several members of the family are commonly isolated from diseased humans or animals, some species are considered true pathogens. The type genus is *Flavobacterium*, Bergey, Harrison, Breed, Hammer and Huntoon 1923, as emended in 1996 (Bernardet *et al.,* 1996).

Other taxa included in the family *Flavobacteriaceae* are the genera *Bergeyella*, *Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Coenonia*, *Empedobacter*, *Gelidibacter*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum*, *Weeksella* and *Zobellia*. Several species unaffiliated to any genus also belong to the family. Several intracellular symbionts of insects and intracellular parasites of amoebae are closely related to the family.

2.2.5. *Methods to study the taxonomy of the Flavobacteriaceae*

2.2.5.1. *Definitions*

Taxonomy refers to the theory and practice of classifying organisms while systematics refers to the study of the diversity of organisms and all relationships among them including their evolutionary relatedness (phylogeny) and all possible biological interactions (Prakash *et al.,* 2007). Taxonomy was traditionally divided into classification, nomenclature and identification of unknown organisms. Phylogeny and population genetics are, however, also necessary to completely define modern biosystematics (Vandamme *et al.*, 1996). Classification is the organisation of large numbers of individual strains into an orderly framework based upon the similarities of their biochemical, physiological, genetic and morphological characteristics. The purpose of classification is to construct

Table 2.2. Currently recognized genera and type species classified in the family *Flavobacteriaceae* (Euzéby, 2012).

Genus	Type species	Source	Reference(s)
Actibacter	*Actibacter sediminis*	Tidal flat sediment	Kim *et al.*, 2008a
Aequorivita	*Aequorivita antarctica*	Under: ice sea water	Bowman and Nichols, 2002
Aestuariicola	*Aestuariicola saemankumensis*	Tidal flat sediment	Kim *et al.*, 2008a
Algibacter	*Algibacter lectus*	Green algae	Nedashkovskaya *et al*., 2007
Aquimarina	*Aquimarina muelleri*	Sea water	Nedashkovskaya *et al*., 2005a
Arenibacter	*Arenibacter latericius*	Marine sediment	Ivanova *et al*., 2001
Bergeyella	*Bergeyella zoohelcum*	Clinical: human, dairy processing environment	Holmes *et al*., 1986b
Bizionia	*Bizionia paragorgiae*	Soft coral	Nedashkovskaya *et al*., 2005b
Capnocytophaga	*Capnocytophaga ochracea*	Clinical: human	Leadbetter *et al*., 1979; Vandamme *et al*., 1996b
Cellulophaga	*Cellulophaga lytica*	Marine environment	Lewin and Lounsberry, 1969; Reichenbach, 1989; Johansen *et al*., 1999
Chryseobacterium	*Chryseobacterium gleum*	Clinical: human, fish, water, milk, marine environment	Holmes *et al*., 1984b
Cloacibacterium	*Cloacibacterium normanense*	Municipal wastewater	Allen *et al.*, 2006
Coenonia	*Coenonia anatina*	Peking duck	Vandamme *et al*., 1999
Costertonia	*Costertonia aggregata*	Marine biofilm	Kwon *et al.*, 2006a
Croceibacter	*Croceibacter atlanticus*	Seawater	Cho and Giovannoni, 2003
Croceitalea	*Croceitalea eckloniae*	Rhizosphere of marine alga	Lee *et al.*, 2008
Dokdonia	*Dokdonia donghaensis*	Sea water	Yoon *et al*., 2005
Donghaeana	*Donghaeana dokdonensis*	Sea water	Yoon *et al*., 2006
Elizabethkingia	*Elizabethkingia meningoseptica*	Blood, clinical specimen, spinal fluid	Kim *et al*., 2005
Empedobacter	*Empedobacter*	Clinical: human	Holmes *et al*., 1978;

	brevis		Holmes *et al*., 1984a; Bernadet *et al*., 1996
Epilithonimonas	*Epilithonimonas tenax*	Hardwater creek	Brambilla *et al*., 2007
Eudoraea	*Eudoraea adriatica*	Sea water	Alain *et al.*, 2008
Flagellimonas	*Flagellimonas eckloniae*	Rhizosphere of *Eckloniae kurome*	Bae *et al*., 2007
Flaviramulus	*Flaviramulus basaltis*	Seafloor basalt	Einen and Øvreas, 2006
Flavobacterium	*Flavobacterium aquatile*	Fresh and salt water, fish, soil	Holmes *et al*., 1984a; Bernardet *et al*., 1996
Formosa	*Formosa algae*	Brown algae	Ivanova *et al*., 2004
Fucobacter	*Fucobacter marina*	Marine environment	Sakai *et al*., 2002
Fulvibacter	*Fulvibacter tottoriensis*	Marine sediment	Khan *et al.*, 2008
Gaetbulibacter	*Gaetbulibacter saemankumensis*	Tidal flat sediment	Jung *et al*., 2005
Gaetbulimicrobium	*Gaetbulimicrobium brevivitae*	Tidal flat sediment	Yoon *et al*., 2006a
Galbibacter	*Galbibacter mesophilus*	Marine sediment	Khan *et al.*, 2007a
Gelidibacter	*Gelidibacter algens*	Sea ice	Bowman *et al*., 1997a
Gillisia	*Gillisia limnaea*	Microbial mats, Antarctica	Van Trappen *et al*., 2004
Gilvibacter	*Gilvibacter sediminis*	Marine sediment	Khan *et al.*, 2007b
Gramella	*Gramella portivictoriae*	Sea urchin	Stanley *et al*., 2005
Jejuia	*Jejuia pallidilutea*	Seawater	Lee *et al*., 2009
Joostella	*Joostella marina*	East Sea (Korea)	Quan *et al*., 2008
Kaistella	*Kaistella koreensis*	Freshwater stream	Kim *et al*., 2004
Kordia	*Kordia algicida*	Red tide	Jae *et al*., 2004
Kriegella	*Kriegella aquimaris*	Marine environment	Nedashkovskaya *et al*., 2008
Krokinobacter	*Krokinobacter genikus*	Marine sediment	Khan *et al*., 2006a
Lacinutrix	*Lacinutrix copepodicola*	Lake: dwelling, calanoid copepod	Bowman and Nichols, 2005
Leeuwenhoekiella	*Leeuwenhoekiella blandensis*	Algal blooms	Pinhassi *et al*., 2006
Leptobacterium	*Leptobacterium flavescens*	Marine sponge and seawater	Mitra *et al*., 2009
Lutaonella	*Lutaonella thermophila*	Coastal hot spring	Arun *et al*., 2009
Lutibacter	*Lutibacter litoralis*	Tidal flat sediment	Choi and Cho, 2006
Lutimonas	*Lutimonas vermicola*	Marine polychaete	Yang *et al*., 2007
Maribacter	*Maribacter sedimenticola*	Marine habitats	Nedashkovskaya *et al*., 2004
Mariniflexile	*Mariniflexile gromovii*	Sea urchin	Nedashkovskaya *et al*., 2006

Marixanthomonas	*Marixanthomonas ophiurae*	Deep:sea brittle star	Romanenko *et al*., 2007
Mesoflavibacter	*Mesoflavibacter zeaxanthinifaciens*	Marine environment	Asker *et al*., 2008
Mesonia	*Mesonia algae*	Green algae	Nedashkovskaya *et al*., 2003a
Muricauda	*Muricauda ruestringensis*	Intertidal sediment	Bruns *et al*., 2001
Myroides	*Myroides odoratus*	Clinical: human	Holmes *et al*., 1977, 1984a; Vancanneyt *et al*., 1996
Nonlabens	*Nonlabens tegetincola*	Microbial mat	Lau *et al*., 2005
Olleya	*Olleya marilimosa*	Particulate material	Mancusa Nichols *et al*., 2005
Ornithobacterium	*Ornithobacterium rhinotracheale*	Respiratory tract of turkey	Vandamme *et al*., 1994b
Persicivirga	*Persicivirga xylanidelens*	Hardwater creek	Brambilla *et al*., 2007
Pibocella	*Pibocella ponti*	Green alga	Nedashkovskaya *et al*., 2005c
Planobacterium	*Planobacterium taklimakanense*	Desert soil	Peng *et al*., 2009
Polaribacter	*Polaribacter filamentus*	Fresh and salt water	Gosink *et al*., 1998
Pseudozobellia	*Pseudozobellia thermophila*	Green alga	Nedashkovskaya *et al*., 2009
Psychroflexus	*Psychroflexus torquis*	Salt water	Bowman *et al*., 1998
Psychroserpens	*Psychroserpens burtonensis*	Salt water	Bowman *et al*., 1997a
Riemerella	*Riemerella anatipesticer*	Clinical and poultry	Segers *et al*., 1993
Robiginitalea	*Robiginitalea biformata*	Marine habitat	Cho and Giovannoni, 2004
Salegentibacter	*Salegentibacter salegens*	Organic water	Dobson *et al*., 1993; McCammon and Bowman, 2000; Suzuki *et al*., 2001
Salinimicrobium	*Salinimicrobium catena*	Saline lake	Lim *et al*., 2008; Chen *et al.*, 2008
Sandarakinotalea	*Sandarakinotalea sediminis*	Marine sediment	Khan *et al.*, 2006b
Sediminibacter	*Sediminibacter furfurosus*	Marine sediment	Khan *et al.,* 2007c
Sediminicola	*Sediminicola luteus*	Marine sediment	Khan *et al.*, 2006c
Sejongia	*Sejongia jeonii*	Moss sample – penguin habitat	Yi *et al.*, 2005
Stanierella	*Stanierella latercula*	Sea water	Nedashkovskaya *et al*., 2005
Stenothermobacter	*Stenothermobacter spongiae*	Marine sponge	Lau *et al*., 2006
Subsaxibacter	*Subsaxibacter broadyi*	Quartz stone cyanobacterial	Bowman and Nichols, 2005

		biofilm	
Subsaximicrobium	*Subsaximicrobium wynnwilliamsii*	Antarctic maritime habitats	Bowman and Nichols, 2005
Tamlana	*Tamlana crocina*	Beach sediment	Lee, 2007
Tenacibaculum	*Tenacibaculum maritimum*	Marine environment	Wakabayashi *et al*., 1986; Bernardet and Grimont, 1989; Suzuki *et al*., 2001
Ulvibacter	*Ulvibacter litoralis*	Green algae	Nedashkovskaya *et al*., 2003b
Vitellibacter	*Vitellibacter vladivostokensis*	Holothurian	Nedashkovskaya *et al*., 2003d
Wautersiella	*Wautersiella falsenii*	Surgical wound	Kämpfer *et al.*, 2006
Weeksella	*Weeksella virosa*	Clinical, human, dairy processing environment	Holmes *et al*., 1986a
Winogradskyella	*Winogradskyella thalassocola*	Algae	Nedashkovskaya *et al*., 2005
Yeosuana	*Yeosuana aromativorans*	Estuarine sediment	Kwon *et al.*, 2006b
Zeaxanthinibacter	*Zeaxanthinibacter enoshimensis*	Marine environment	Asker *et al*., 2007
Zhouia	*Zhouia amylolytica*	Marine sediment	Liu *et al*., 2006
Zobellia	*Zobellia galactanivorans*	Marine environment	Barbeyron *et al*., 2001
Zunongwangia	*Zunongwangia profunda*	Deep-sea sediment	Qin *et al*., 2007; Euzéby, 2007

homogeneous groups which consist of descendents of the nearest common ancestor (Prakash *et al.,* 2007). Nomenclature is the naming of individual groups in the framework with a binomial name according to strict rules. Identification is the determination of discriminating properties for rapid recognition of new isolates. Phylogeny and population genetics involve the creation of a satisfactory phylogenetic and evolutionary framework (Gevers *et al.,* 2006).

In the past, taxonomists used monophasic approaches such as the ancient “form” classification and pathovar systems (Vandamme *et al.,* 1996). These were based on traits such as shape, colour, size, staining properties, motility, host range, pathogenecity and assimilation of a few carbon sources (Prakash *et al.,* 2007). Developments in bacterial taxonomy led to a consensus type of taxonomy integrating different kinds of data and information from phenotypic, genotypic and phylogenetic aspects of microorganisms. The term “polyphasic taxonomy” was

coined by Colwell (1970) and a polyphasic approach to bacterial systematics was progressively adopted by most bacteriologists. It integrates phenotypic and chemotaxonomic characterization with genomic and phylogenetic data (Murray *et al.,* 1990; Vandamme *et al.,* 1996; Bernardet *et al.,* 2002). Genotypic methods directed towards DNA or RNA molecules dominate modern taxonomy (Vandamme *et al.,* 1996). However, any species classified according to phylogenetic similarities must show phenotypic consistency (Wayne *et al.,* 1987). Phenotypic features are derived from proteins and their functions, different chemotaxonomic markers and a wide range of other expressed features (Vandamme *et al.,* 1996).

The species is the basic unit of bacterial taxonomy. It is defined as a group of strains, including the type strain, sharing 70% or greater DNA relatedness with 5% or less ΔT_m (Wayne *et al.,* 1987). T_m is the melting temperature of the hybrid as determined by stepwise denaturation and ΔT_m is the difference in T_m in degrees Celcius between the homologous and heterologous hybrids formed under standard conditions. Phenotypic and chemotaxonomic features should agree with this definition (Wayne *et al.,* 1987; Vandamme *et al.,* 1996). This is further corroborated from data on 16S rRNA gene sequence analysis wherein bacterial strains showing more than 3% divergence are considered to be members of a different species (Stackebrandt and Goebel, 1994; Vandamme *et al.,* 1996; Clarridge, 2004; Gevers *et al.,* 2005; Coenye *et al.,* 2005). Figure 2.2 shows the taxonomic resolution of some of the currently used polyphasic taxonomy techniques. The important taxonomical techniques will now be discussed briefly:

2.2.5.2. *Genotypic methods*

a.) *Pulsed Field Gel Electrophoresis (PFGE)*

Pulsed Field Gel Electrophoresis (PFGE) uses *in situ* lysis of bacterial whole cells in agarose plugs followed by restriction of the DNA using specific enzymes. The digested bacterial plugs are positioned in agarose gels and subjected to electrophoresis under current polarity that is changed at specific intervals of time

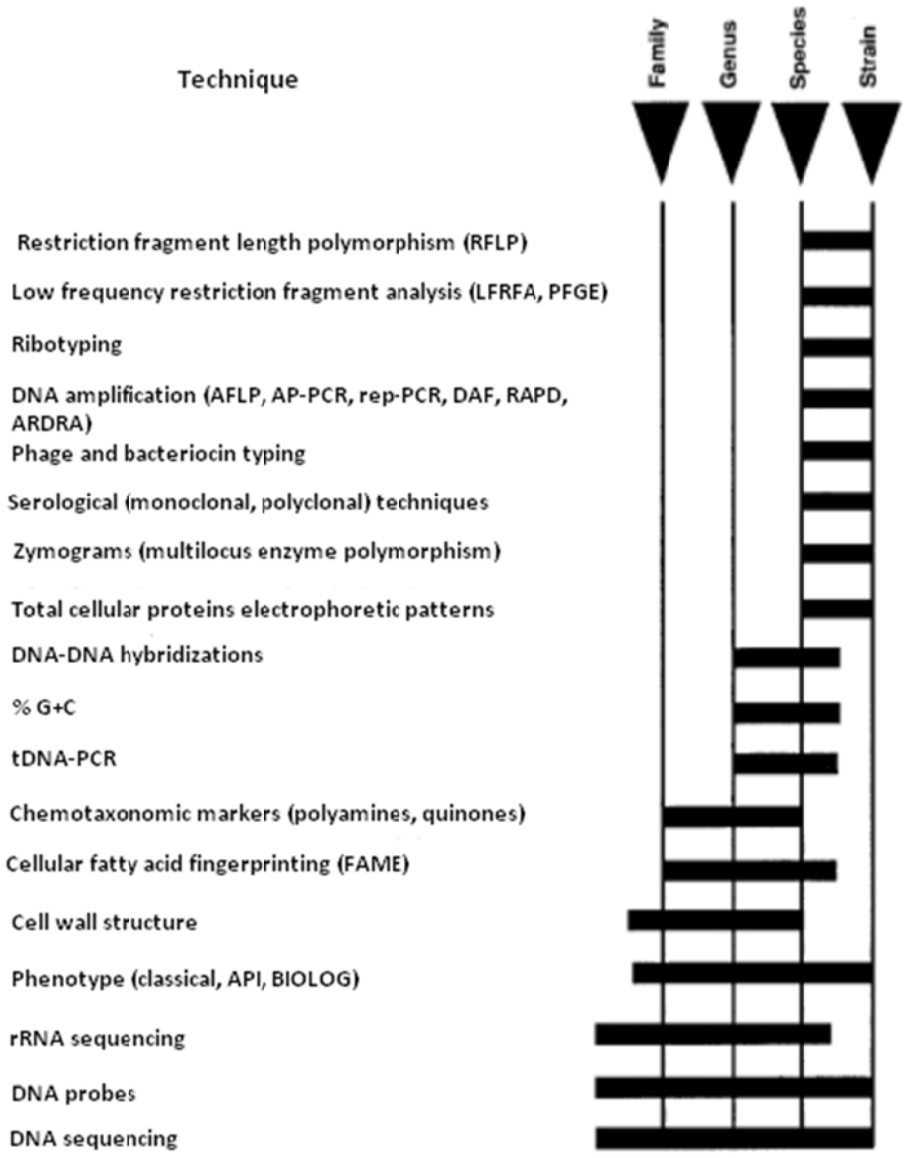

Fig. 2.2. Taxonomic resolution of some of the currently used techniques (Vandamme *et al.,* 1996). Abbreviations: AFLP, amplified fragment length polymorphism; AP-PCR, arbitrarily primed PCR; ARDRA, amplified rDNA restriction analysis; DAF, DNA amplification fingerprinting; FAMEs, fatty acid methyl esters; LFRFA, low frequency restriction fragment analysis; LMW, low molecular weight; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; rep-PCR, repetitive element sequence-based PCR; RFLP, restriction fragment length polymorphism; tDNA-PCR, transfer DNA intergenic spacer PCR; 1D, 2D, one and two-dimensional, respectively.

(Prakash *et al.,* 2007). This method can resolve very large DNA fragments (10 to 800 Kb in size) which are visualised by staining (Prakash *et al.,* 2007). Protein bands that are formed are compared and patterns are usually similar for species. Pot *et al.* (1994) reported that strains with at least 70% DNA binding values tend to display similar protein fingerprints, with only minor differences.

b.) *Restriction Fragment Length Polymorphism (RFLP) and Plasmid DNA profiling*

These techniques are based on the random distribution of restriction sizes in the genome and are preliminary typing methods that generate restriction profiles of the microbe DNA, for RFLP, and the plasmid, for plasmid profiling (Prakash *et al.,* 2007). The types of profiles produced are dependent upon the bacterial group that is investigated and the type of restriction enzyme used. The disadvantage of the RFLP technique is that its profiles are complex and difficult to compare while plasmid profiling has the shortcoming of generating profiles that may not be consistent since it is difficult for bacteria to maintain plasmids over several generations (Regnault *et al.,* 1997).

c.) *RFLP Derivative Methods*

Other methods like ribotyping, amplified ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) are derivatives of RFLP (Prakash *et al.,* 2007).

Ribotyping involves the use of rRNA, rDNA or gene-specific oligonucleotides as probes against enzyme restricted DNA to generate complex profiles in a process that is automated and read using riboprinters (Regnault *et al.,* 1997).

ARDRA can be used to screen large numbers of isolates simultaneously. The technique employs the digestion of amplified ribosomal DNA using different restriction enzymes to produce patterns which are combined to obtain a profile (Maslow *et al.,* 1993).

In RFLP, specific adapters are ligated to the enzyme restricted DNA which is then amplified using primers from the adapter and restriction site-specific sequences (Prakash *et al.,* 2007).

RAPD is also known as Arbitrary Primed PCR (AP-PCR). Short primer sequences, octa- to decamer, randomly anneal to genomic DNA and initiate amplification. A PCR product is formed if the primers anneal in proper orientation such that the distance between the annealing sites is a few kilobases apart. A number of amplified fragments are formed which when resolved on the gel, generates a strain-specific profile (Olive and Bean, 1999; Czekajlo *et al.,* 2006).

Repetitive PCR (Rep-PCR) is another DNA amplification based technique for bacterial resolution up to strain level (Versalovic *et al.,* 1994). Its principle is based upon the amplification of naturally occurring, highly conserved and repetitive DNA sequences, which are present in multiple copies throughout the genomes of most bacteria (Lupski and Weinstock, 1992).

d.) *16S rRNA gene Sequencing*

The 16S rRNA technique is indispensable in bacterial taxonomy (Vandamme *et al.,* 1996). This method is used for making phylogenetic comparisons up to the genus level based on the conserved part of the genome (Woese *et al.,* 1987; Clarridge, 2004). All the three kinds of rRNA molecules (5S, 16S, 23S) and spacers between them can be used for phylogenetic analysis. The 16S rRNA gene (1650 bp) is mainly used because of its appropriate intermediate size. The 5S rRNA gene (120 bp) is small while the 23S rRNA gene (3300 bp) is large (Amann *et al.,* 1995; Mora and Amann, 2001). The 16S rRNA gene sequence has the advantage that it is distributed universally, has a highly conserved nature, plays a pivotal role in protein synthesis, cannot be transferred horizontally and has an evolution rate which represents an appropriate level of variation between organisms (Woese, 1987; Stackebrandt and Goebel; 1994; Mora and Amann, 2001; Clarridge, 2004). Strains showing less than 97% 16S rRNA gene sequence similarity to all known taxa are considered to belong to a new species, as there

are hardly any examples in which strains with this extent of divergence in 16S rRNA gene sequence are defined as one species (Rossello-Mola and Amann, 2001).

A phylogenetic tree or dendrogram is constructed and used to ascertain the genus to which the strain belongs and its closest neighbours (Prakash *et al.,* 2007). Bacterial strains exhibiting more than 3% on 16S rRNA gene sequence divergence are considered to be members of different species (Stackebrandt and Goebel; 1994; Vandamme *et al.,* 1996; Clarridge *et al.,* 2004; Coenye *et al.,* 2005; Gevers *et al.,* 2005). Strains with 97% 16S rRNA gene sequence similarity to those found in the GenBank are assumed to be members of that genus (Gillis *et al.,* 2001). However, the 16S rRNA gene sequence alone cannot be used to delineate species within certain groups such as the flavobacteria and additional DNA-DNA hybridizations are often required (Stackebrandt and Goebel; 1994; Gillis *et al.,* 2001). The 16S rRNA gene sequences have high levels of variation, even between strains of the same species due to, among others, inter-operon differences (Clayton *et al.,* 1995; Hankka, 1996).

e.) *DNA-DNA Hybridization*

The DNA-DNA hybridization or DNA-DNA reassociation technique is applied in classification for delineation of species. It compares the whole genome between two bacterial species. A committee on systematics recommended that bacterial species generally would include strains with 70% or greater DNA-DNA relatedness and with 5% or less ΔT_m values and both values must be considered (Wayne *et al.,* 1987). Deoxyribose nucleic acid (DNA) can be denatured at high temperatures and the whole molecule can be brought back to its native state by lowering down the temperature (reassociation). This technique is based on three parameters namely: G+C mol%, ionic strength of the solution and the melting temperature (T_m) of the DNA hybrid (Prakash *et al.,* 2007). The melting point (T_m) and mol% G+C are linearly related and yield information on the temperature at which the two DNA strands are separated (Jay, 2000). The more the similarity between the heteroduplex molecule, the more the temperature will be required to

separate it and therefore the higher the T_m value. The stringency of this technique is dependent upon the salt and formamide concentration (Prakash *et al.,* 2007).

The DNA-DNA hybridization techniques have shortfalls in their high experimental error, inaccurate reproducibility of the result and dependence on physicochemical parameters (Grimont *et al.,* 1980). They have failed to generate a cumulative database, they are cumbersome, and require great quantities of DNA (Stackebrandt and Goebel; 1994; Vandamme *et al.,* 1996; Mora, 2006). Another shortfall is that the DNA-DNA hybridization techniques give the relative percent of similarity but not the actual sequence identity (Prakash *et al.,* 2007).

f.) *Guanine and Cytosine Ratio (G+C Ratio)*

Each species has a specific amount of guanine and cytosine expressed as mole percent guanine and cytosine (mol% G+C). Analysis of DNA G+C ratio is one of the classical genotypic methods in classification (Prakash *et al.,* 2007). In well defined species the G+C content of strains usually differ by less than 3 Mol%, while in well defined genera species differ by less than 10 Mol%. The mol% G+C ranges between 24 and 76% in bacteria (Prakash *et al.,* 2007).

2.2.5.3. *Chemotaxonomic methods*

Vandamme *et al.* (1996), and Mora and Amann (2001) described chemotaxonomy as the application of analytical methods for collecting information on different chemical constituents or chemotaxonomic markers of bacterial cells in order to group them into different taxonomic ranks. This is possible because the markers are distributed unevenly among different microbial groups. The most commonly used markers include cell wall and cell membrane components such as peptidoglycan, teichoic acids, lipopolysaccharides, polar lipids, fatty acids (both composition and relative ratio), isoprenoid quinones, and polyamines (Busse and Auling, 1988; Suzuki *et al.,* 1993). Respiratory quinones and whole cell fatty acids are the two analyses mostly applied to the genus *Chryseobacterium* and will now be discussed in more detail.

Respiratory quinones are important chemotaxonomic markers. They belong to a class of terpenoid lipids and they are constituents of bacterial plasma membranes. Members of the family *Flavobacteriaceae* exhibit menaquinone 6 as their only or major respiratory quinone (Bernardet *et al.,* 1996; 2002), whereas menaquinone 7 is found in members of related families (Hanzawa *et al.,* 1995; Bernardet *et al.,* 2002). In other bacterial groups, this technique can delineate bacteria up to the genus level (Vandamme *et al.,* 1996; Gillis *et al.,* 2001; Prakash *et al.,* 2007).

Fatty acids are present in bacterial cells and their composition provides high quality information that is useful in both taxonomic studies and identification analyses (Vandamme *et al*., 1996). They are major constituents of lipids and lipopolysaccharides. Bacterial membranes are mainly composed of polar lipids while other types of lipids such as sphingophospholipids occur only in a few taxa.

Lipopolysaccharides present in the outer membranes of Gram-negative bacteria can be analyzed by gel electrophoresis, giving typical lipopolysaccharide ladder patterns which are interpreted as variants in the O-specific side chains. The variability in chain length, double bond position and substituent groups of the more than 300 different fatty acid chemical structures is used in the characterization of bacterial taxa (Suzuki *et al.,* 1993). In some genera, whole-cell fatty acid analysis can delineate individual species or subspecies, while in other genera, different species have identical fatty acid profiles (Welch, 1991).

Cellular fatty acid methyl ester content is a stable parameter when highly standardized conditions are used (Vandamme *et al*., 1996). Cellular fatty acid analysis is useful as a quick and rather inexpensive, fairly simple and highly automated screening method which allows the comparison and clustering of large numbers of strains (Vandamme *et al*., 1996).

The predominant fatty acids found in members of the family *Flavobacteriaceae* are usually characteristic of genera though some fatty acid profiles help to differentiate species provided that standardized culture conditions are employed (Barbeyron *et al.,* 2001; Bernardet and Nakagawa, 2006). Hugo *et al.* (1999)

noted that *Chryseobacterium* species could not be differentiated on the basis of fatty acid profiles, whilst those of the related species *Elizabethkingia meningoseptica*, *Bergeyella zoohelcum* and *Empedobacter brevis* are distinct.

Fatty acids that are common to *Chryseobacterium* are the branched-chain fatty acids 15:0 iso, iso 17:1 ω9c, 17:0 iso 3OH, and summed feature 4 (15:0 iso 2OH or 16:1 ω7t or both]). In their recent proposal for reclassification of *Sejongia* species within *Chryseobacterium* following a review of polyphasic taxomic data, Kämpfer *et al.* (2009a) reported that all three currently known *Sejongia* species reveal iso 15:0, anteiso-15:1 and iso 17:1ω9c as the major fatty acids which is in agreement with fatty acid patterns of *Chryseobacterium*. *Sejongia antarctica*, *Sejongia jeonii* and *Sejongia marina* were reclassified in the genus *Chryseobacterium* as the new combinations *Chryseobacterium antarcticum* comb. nov., *Chryseobacterium jeonii* comb. nov. and *Chryseobacterium marinum* comb. nov. Therefore, although fatty acid profiles of *Chryseobacterium* are not species specific, their determination assists in assignment of the isolates to the genus.

2.2.5.4. Phenotypic Methods

The report of the Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics stressed that any phylogenetically based taxonomic scheme must also show phenotypic consistency (Wayne *et al.,* 1987). The phenotype is the observed expression of the genotype. It includes morphological, physiological and biochemical properties of the organism (Prakash *et al.,* 2007). In the past, taxonomists relied on imperative studies of phenotypic features for bacterial taxonomy (Prakash *et al.,* 2007) and current routine microbiology methods for microbial identifications rely almost exclusively on these differential phenotypic features (Vandamme *et al.,* 1996). Differential characteristics of some taxa classified in the family *Flavobacteriaceae* are as shown later in Table 2.4.

Classical analyses that are done include characteristics of the organism on different growth substrates, growth range of microorganisms on different conditions of salt, pH and temperature, and susceptibility towards different kinds

of antimicrobial agents (Prakash *et al.,* 2007). The ability to produce specific metabolites and the production of enzymes also give valuable information on potential spoilage abilities such as proteolysis and lipolysis. The production of gas and metabolites containing alcoholic compounds and indole contribute to certain flavour compounds (Banwart, 1989).

Flavobacteriaceae have different enzymatic abilities and many of them can degrade different kinds of organic macromolecules (Reichenbach, 1989). Enzymatic screenings performed on environmental isolates aimed at discovering potentially useful exoenzymes resulted in the description of several new taxa (Yamaguchi and Yokoe, 2000; Barbeyron *et al.,* 2001; Humphry *et al.,* 2001; Sakai *et al.,* 2002). Major available biopolymers found in the different habitats influence the types of various enzymes produced by *Flavobacterium* (Kirchman, 2002). Thus, soil and freshwater *Flavobacterium* frequently produce cellulases, pectinases, xylanases and chitin degrading enzymes that decompose moribund tissue, fungi and insects (Reichenbach, 1989; Haack and Breznak, 1993; Cottrell and Kirchman, 2000; Johansen and Binnerup, 2002). Marine flavobacteria usually produce enzymes that degrade agars, laminarin, xylan, fucoidan, and carrageenans from micro- or macro-algae (Johansen *et al.*, 1999; Barbeyron *et al.,* 2001; Humphry *et al.,* 2001; Sakai *et al.,* 2002).

Flavobacteria from various environments are also capable of producing proteolytic enzymes that probably play an important role in the virulence of some of the pathogenic strains (Bernardet and Nakagawa, 2006). Other members produce keratinolytic enzymes that may have important uses in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes (Shih, 1993, Onifade *et al.,* 1998; Riffel *et al.*, 2003; Casarin *et al.,* 2008). Degradation of crystalline cellulose (filter paper) is of particular taxonomic significance since it requires the production of a specific cellulase and hence only strains that degrade filter paper should be regarded as cellulose degraders (Reichenbach, 1989). Cellulose degradation distinguishes members of the family *Flavobacteriaceae* from those of the genus *Cytophaga*, now restricted to cellulolytic organisms (Nakagawa and Yamasato, 1996).

The use of phenotypic fingerprinting systems such as Biolog and API is increasing. However, their inclusion in official descriptions restricts the examination of bacterial phenotype to a minimum, thus also restricting the knowledge of the phenotype (Vandamme *et al.,* 1996). Another shortcoming of phenotypic methods is the problem of reproducibility of results between different laboratories. It is, therefore, recommended that only standardized procedures should be used during execution of experiments (On and Holmes, 1991; 1992). Another major disadvantage with phenotypic methods is the conditional nature of gene expression. The same organism might show different phenotypic characteristics in different environmental conditions. A comparison of the phenotypic data with similar a set of data from the type strain(s) of closely related organism(s) should be made (Prakash *et al.,* 2007).

a) The Omnilog System

The Omnilog identification and phenotype MicroArray systems are products of Biolog, Inc. which greatly simplify testing of cells. The principle behind these technologies is tetrazolium dye that changes colour when microbial (or mammalian) cells metabolize substances that promote respiration. This dye is incorporated in a 96-well microplate (BIOLOG, 2008). The redox chemistry method measures cellular metabolism, providing utility for two distinct areas:

b) Microbial identification

The Omnilog system can be used to identify and characterize a wide range of organisms. Environmental and pathogenic micro-organisms produce a characteristic pattern or “metabolic fingerprint” from discrete test reactions within the 96-well microplate. These patterns are then analyzed with sophisticated interpretation software and compared to extensive organism and pathogen databases. Manual systems for the Gen I and Gen II identification systems involve visual reading of Gram-positive and Gram-negative microplates and

manual reaction entry. In the semi-automated system, plates are read by a Microstation coupled to a data base. In the fully automated system, the Omnilog, plates are read by the Omnilog incubator. The developed metabolic fingerprints are automatically read and recorded in seconds. This eliminates the subjectivity of visual interpretation. The patterns are then compared to an extensive database for final identification. It can identify over 1,900 species of aerobic bacteria, anaerobic bacteria, yeast, and filamentous fungi.

c) Characterization of cellular functions (Omnilog Combo system)

The OmniLog Phenotype MicroArrays™ (PM) system for bacteria consists of 20 x 96-well plates with about 200 C-sources, 400 N-sources, 100 P- and S-sources, 100 nutrient supplements, and a range of conditions varying the pH, ion and osmotic status of the culture environment. This enables the researcher to see what stimulates growth and, equally important, what inhibits growth (Bochner *et al.*, 2008). Phenotype MicroArrays™ are a breakthrough platform technology for measuring cell pathway activities and phenotypes by analyzing cells under thousands of culture conditions. Through amplification and precise quantification of phenotypes, researchers are able to obtain an unbiased perspective of the effect on cells of genetic differences, environmental change, and exposure to chemicals or drugs. Panels of up to 1920 phenotypic tests have been developed for bacterial and fungal cells, as well as mammalian cells (BIOLOG, 2008).

Phenotype Microarrays represent the third major technology, alongside DNA Microarrays and Proteomic technologies, that is needed in the genomic era of research and drug development. Just as DNA Microarrays and Proteomic Technologies have made it possible to assay thousands of genes or proteins all at once, PMs make it possible to quantitatively measure thousands of cellular phenotypes all at once.

2.2.6. *Procedure for Polyphasic Taxonomy*

Classifying 100 strains of unknown possible identity, presents totally different problems compared to classifying a single unidentified *Bordetella* strain and hence there are no simple and straightforward guidelines for performing polyphasic taxonomic studies (Vandamme *et al.,* 1996). Figure 2.3 depicts the step-by-step procedure for taxonomical characterization of newly isolated strains.

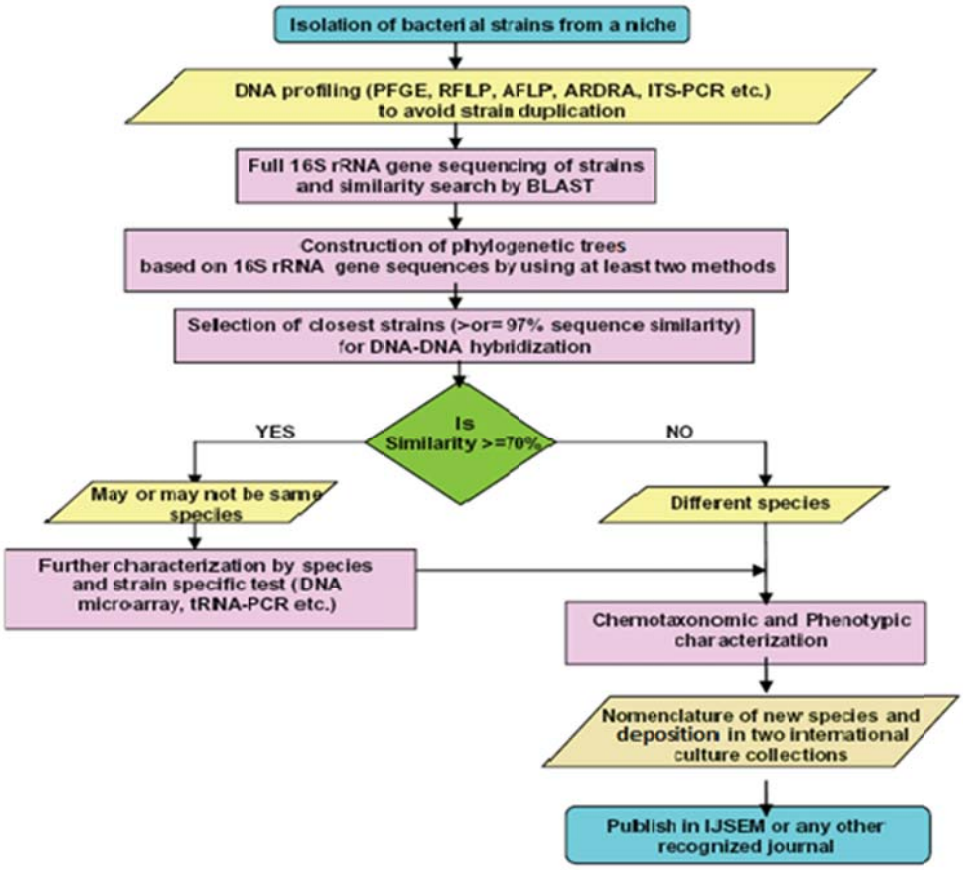

Fig. 2.3. The step-by-step procedure for taxonomical characterization of newly isolated strains (Prakash *et al.,* 2007).

Any chosen strategy may be influenced during the course of the study by the results obtained. Preferably, two different screening techniques should be

available to compare and group large numbers of strains (Vandamme *et al.,* 1996).

The genotypic structure of such groups may be analysed by DNA-based typing methods, but ultimately, DNA-DNA hybridization studies of a limited number of well-chosen strains are required to delineate individual species. Phenotypic and chemotaxonomic characterization should then be done (Vandamme *et al.,* 1996), followed by nomenclature and submission to two international culture collections and finally publication in the International Journal of Systematic and Evolutionary Microbiology (IJSEM; Prakash *et al.,* 2007).

2.3. The genus *Chryseobacterium*

2.3.1. *Taxonomy*

The long established *Flavobacterium* genus was ill defined and relied on parameters which are now considered to be of little taxonomic importance. It comprised of a collection of predominantly yellow-pigmented organisms. According to modern genotypic standards, these organisms are not related. Consequently, the genus *Flavobacterium* has been restricted and redefined many times from the time it was created in 1923 to accommodate Gram-negative, non-spore forming, yellow pigmented rods, that produce acids from carbohydrates weakly resulting in the reclassification of most erstwhile flavobacteria (Holmes and Owen, 1979; Vandamme *et al.,* 1994a; Bernardet *et al.,* 1996). The genus *Chryseobacterium*, together with the genera *Bergeyella* and *Empedobacter*, was built on the ruins of the genus *Flavobacterium* shortly after the latter was thoroughly emended following extensive phylogenetic investigations (Vandamme *et al.,* 1994a; Bernardet *et al.,* 1996; 2006). It was proposed by Vandamme *et al.* (1994a) to include six former *Flavobacterium* species in the new *Chryseobacterium* genus which at the time of the delineation comprised of *Chryseobacterium balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, [*C.*] *meningosepticum* and *C. scophthalmum*. *Chryseobacterium gleum* was proposed as the type species (Vandamme *et al.,* 1994a).

Kim *et al.* (2005a) proposed the splitting of the genus *Chryseobacterium* and the transfer of organisms previously known as *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to a new genus, *Elizabethkingia* under the new epithets *Elizabethkingia meningoseptica* and *Elizabethkingia miricola.* The split was a consequence of DNA-rRNA hybridizations that were extensively used for phylogenetic investigations of the genus *Flavobacterium* and related genera in the 1980s and 1990s (Bauwens and De Ley, according to Bernardet *et al.,* 2006; Segers *et al.,* 1993b; Mudarris *et al.,* 1994; Vandamme *et al.,* 1994a; Vancanneyt *et al.,* 1996) and the use of high quality 16S rRNA gene sequencing which further clarified the separate position of *Elizabethkingia meningoseptica* as depicted in Figure 2.4, which shows the phylogenetic relationships among representatives of the genera *Chryseobacterium* and *Elizabethkingia* (Bernardet *et al.*, 2006).

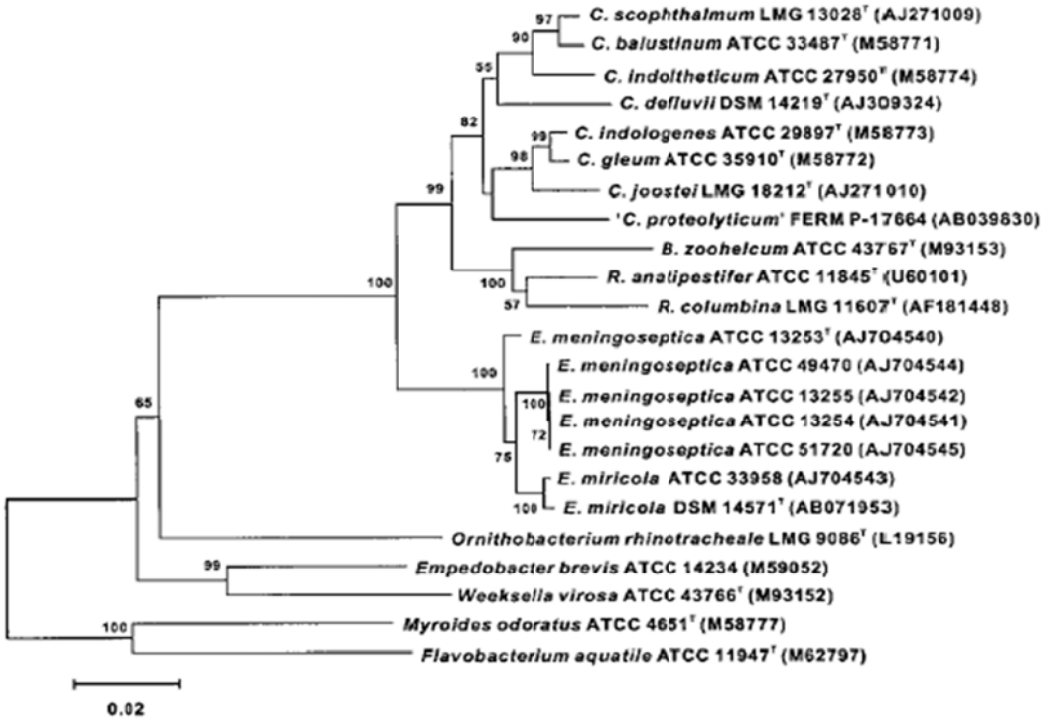

Fig. 2.4. 16S rRNA gene sequence dendrogram obtained by distance matrix (neighbour joining) analysis, showing the positions of the seven strains of *Elizabethkingia*. Species of some genera within the family *Flavobacteriaceae* were used to define the root. Numbers at branching points refer to bootstrap values. Bar, 2 substitutions per 100 nucleotide positions. Abbreviations: *C*., *Chryseobacterium*; *B*., *Bergeyella*; *E*., *Elizabethkingia*; *R*., *Riemerella*.

Many new *Chryseobacterium* species have been described since the genus was established. These include: *C. defluvii*, isolated from sewage water (Kämpfer *et al.,* 2003); *C. joostei*, isolated from raw milk (Hugo *et al.,* 2003); *C. formosense*, isolated from garden lettuce (Young *et al.,* 2005); *C. daecheongense*, isolated from freshwater lake sediment (Kim *et al.,* 2005b); *C. taechungense*, isolated from contaminated soil (Shen *et al.,* 2005) and *C. shigense*, isolated from a lactic acid beverage (Shimomura *et al.,* 2005). "*Chryseobacterium proteolyticum*" was described by Yamaguchi and Yokoe (2000) but has not been validly published (Bernardet *et al.,* 2002).

Currently recognised species of the genus *Chryseobacterium* as well as those of the genus *Elizabethkingia* and their sources of isolation are shown in Table 2.3. The number of new species grew rapidly in a very short time and it is likely to keep growing since many organisms phylogenetically related to the genus *Chryseobacterium* have already been reported from investigations of various environments using both culture-dependent and -independent methods (Bernardet *et al.,* 2009). Recently, Kämpfer *et al.* (2009a) proposed to reclassify *Sejongia antarctica*, *Sejongia jeonii* and *Sejongia marina* in the genus *Chryseobacterium* as the new combinations *Chryseobacterium antarcticum* comb. nov., *Chryseobacterium jeonii* comb. nov. and *Chryseobacterium marinum* comb. nov. on the basis that 16S rRNA analysis of the type strains of the currently known *Sejongia* species fell in the *Chryseobacterium* cluster at a depth similar to other *Chryseobacterium* species groups. Moreover, *Sejongia* and *Chryseobacterium* also showed strong congruence in phenotypic features.

Similarly, Kämpfer *et al.* (2009b) also proposed to reclassify the known *Kaistella* species, *Kaistella koreensis* in the genus *Chryseobacterium* as the new combination *Chryseobacterium koreense* comb. nov.

Table 2.3. Species classified in the genera *Chryseobacterium* and *Elizabethkingia* and their sources of isolation.

Species	G + C (mol %)	Source	Reference(s)
C. antarcticum	34.0	Antarctic soil	Yi *et al*., 2005; Kämpfer *et al*., 2009a
C. anthropi		Human clinical specimens	Kämpfer *et al.,* 2009b
C. aquaticum	38.3-38.5	Water reservoir	Kim *et al*., 2008
C. aquifrigidense	35.6	Water-cooling system	Park *et al*., 2008
C. arothri	36.5	Pufferfish *Arothron hispidus*	Campbell *et al*., 2008
C. arthrosphaerae	Not determined	Faeces of the pill millipede *Arthrosphaera magna* Attems (India)	Kämpfer *et al*., 2010
C. balustinum	33.0	Heart blood of fresh water fish (dace, *Leuciscus leuciscus*)	Holmes *et al.,* 1984a
C. bovis	38.6	Raw cow's milk	Hantsis-Zacharov *et al*., 2008a
C. caeni	38.2	Bioreactor sludge	Quan *et al*., 2007
C. chaponense	Not determined	Atlantic salmon (*Salmo salar*), (Lake Chapo, Chile)	Kämpfer *et al*., 2011
C. culicis	Not determined	Midgut of the mosquito *Culex quinquefasciatus*	Kämpfer *et al*., 2010
C. daecheongense	37.0	Lake Daecheong sediment	Kim *et al.,* 2005b
C. daeguense	36.8	Wastewater of a textile dye works	Yoon *et al*., 2007
C. defluvii	Not determined	Activated sludge	Kämpfer *et al.,* 2003
C. elymi	36.9	Rhizosphere of coastal sand dune plants (Chungnam Province, Korea Republic)	Cho *et al*., 2011
C. flavum	37.2	Polluted soil	Zhou *et al*., 2007
C. formosense	Not determined	Rhizosphere of lettuce	Young *et al.,* 2005
C. gambrini	37.8	Beer-bottling plant	Herzog *et al*., 2008
C. ginsenosidimutans	35.7	Soil of a *Rhus vernicifera*-cultivated field	Im *et al*., 2011
C. gleum	37.0	Human vaginal swab	Holmes *et al.,* 1984b
C. greenlandense	39.6-41.6	Deep Greenland ice	Loveland-Curtze *et*

		core	*al*., 2010
C. gregarium	38.4	Decaying plant material	Behrendt *et al*., 2008
C. hagamense	Not determined	Rhizosphere of coastal sand dune plants (Chungnam Province, Korea Republic)	Cho *et al*., 2011
C. haifense	37.8	Raw milk	Hantsis-Zacharov and Halpern, 2007
C. hispanicum	34.3	Drinking water distribution system	Gallego *et al*., 2006
C. hominis	36.5	Clinical isolate	Vaneechoutte *et al*., 2007
C. humi	34.0	Industrially contaminated sediments, Portugal	Pires *et al*., 2010
C. hungaricum	37.5	Hydrocarbon-contaminated soil	Szoboszlay *et al*., 2008
C. indologenes	38.0	Human trachea at autopsy	Yabuuchi *et al.,* 1983
C. indoltheticum	34.0	Marine mud	Bernardet *et al.,* 2006
C. jejuense	41.4	Soil	Weon *et al*., 2008
C. jeonii	36.0	Antarctic moss	Yi *et al*., 2005 Kämpfer *et al*., 2009a
C. joostei	37	Raw cow's milk	Hugo *et al.,* 2003
C. koreense	Not determined	Human clinical specimens	Kämpfer *et al.,* 2009b
C. lathyri	36.6	Rhizosphere of coastal sand dune plants (Chungnam Province, Korea Republic)	Cho *et al*., 2011
C. luteum	Not determined	Phyllosphere of grasses	Behrendt *et al*., 2007
C. marinum	35.0	Antarctic seawater	Lee *et al*., 2007 Kämpfer *et al*., 2009a
C. molle	39.2	Beer-bottling plant	Herzog *et al*., 2008
C. oranimense	Not determined	Raw cow's milk	Hantsis-Zacharov *et al*., 2008b
C. pallidum	38.1	Beer-bottling plant	Herzog *et al*., 2008
C. palustre	43.0	Industrially contaminated sediments, Portugal	Pires *et al*., 2010
C. piperi	38.6	Freshwater creek in north-central Pennsylvania	Strahan *et al*., 2011
C. piscicola	32.3-32.5	Diseased salmonid fish	Ilardi *et al*., 2009
C. piscium	33.6	Fish	de Beer *et al*., 2006
C. rhizosphaerae	35.9	Rhizosphere of	Cho *et al*., 2011

		coastal sand dune plants (Chungnam Province, Korea Republic)	
"C. proteolyticum"	37.0	Soil, rice field	Yamaguchi and Yokoe, 2000
C. scophthalmum	34.0	Gills of diseased turbot (*Scophthalmus maximus*)	Mudarris *et al.,* 1994
C. shigense	36.6	Lactic acid beverage	Shimomura *et al*., 2005
C. soldanellicola	28.8	Roots of sand-dune plants	Park *et al*., 2006a
C. soli	39.9 and 41.4	Soil samples	Weon *et al*., 2008
C. solincola	40.9	Soil, Western Algeria	Benmalek *et al*., 2010
C. taeanense	32.1	Roots of sand-dune plants	Park *et al*., 2006b
C. taichungense	Not determined	Tar-contaminated soil	Shen *et al.,* 2005
C. taiwanense	36.8	Soil	Tai *et al*., 2006
C. treverense	Not determined	Human blood, Treves (Trier, West Germany)	Yassin *et al*., 2010
C. ureilyticum	36.4	Beer-bottling plant	Herzog *et al*., 2008
C. vrystaatense	37.1	Chicken-processing plant	de Beer *et al*., 2005a
C. wanjuense	37.8	Greenhouse soil	Weon *et al*., 2006
C. xinjiangense	33.5	Soil, Xinjiang Province, China	Zhao *et al*., 2011
C. yonginense	31.3	Mesotrophic artificial lake, Yongin, Korea	Joung and Joh, 2011
E. miricola	35.0	Condensation water, space station Mir, 1997	Kim *et al.,* 2005a
E. meningoseptica	37.0	Human cerebrospinal fluid	King, 1959 Holmes *et al.,* 1984a Kim *et al.,* 2005a

A new species, *Chryseobacterium anthropi* sp. nov.*,* was proposed to accommodate clinical isolates biochemically similar to *Kaistella koreensis* (Kämpfer *et al.,* 2009b)*.* In another study, Kämpfer *et al*. (2009c), compared the type strains of *Chryseobacterium arothri* (DSM 19326^T) and *C. hominis* (NF 802^T). The strains showed 99.9% identity in 16S rRNA gene sequences while DNA-DNA hybridization studies showed 100% (reciprocal, 76%) relatedness. All phenotypic data showed no pronounced differences. On the basis of these results, they proposed the reclassification of *Chryseobacterium arothri* Campbell *et al*. 2008 as a later heterotypic synonym of *Chryseobacterium hominis* Vaneechoutte *et al*. 2007. Therefore, at present there are 58 *Chryseobacterium* species in the *List of Prokaryotic Names with Standing in Nomenclature* since "*Chryseobacterium proteolyticum*" has not been validly published (Euzéby, 2012).

2.3.2. *Description of the genus Chryseobacterium*

The following is the most recent description of the genus as given by Vandamme *et al*. (1994) and as emended by Kämpfer *et al*. (2009b).

Genus X. *Chryseobacterium* (Effective publication: Vandamme, Bernardet, Segers, Kersters and Holmes 1994, 829 ^{VP}) emend. Kämpfer, Vaneechoutte, Lodders, de Baere, Avesani, Janssens, Busse, and Wauters 2009.

(Chry.se.o.bac.te'ri.um. Gr. adj. *chryseos*, golden; Gr. neut. n. *bakterion*, a small rod; N. L. neut. n. *Chryseobacterium*, a yellow rod)

Cells are Gram-negative, non-motile, non spore forming rods with parallel sides and rounded ends; typically the cells are 0.5 μm wide and 1 to 3 μm long. Intracellular granules of poly-β-hydroxybutyrate are absent. Aerobic. Chemoorganotrophic. All strains grow at 30 ^oC; most strains grow at 37 ^oC. Growth on solid media is typically pigmented (yellow to orange), but non-pigmented strains occur.

Colonies are transluscent (occasionally opaque), circular, convex or low convex, smooth and shiny, with entire edges. Positive for catalase, oxidase and

phosphatase activities. Several carbohydrates including glycerol and trehalose, are oxidized. Agar is not digested. Resistant to a wide range of antimicrobial agents. Additional features are shown in Table 2.4.

Branched-chain fatty acids (i.e., 15:0 iso, iso 17:1 ω9c, 17:0 iso 3OH, and summed feature 4 [15:0 iso 2OH or 16:1 ω7t or both]) are predominant (Segers *et al.,* 1993b). Sphingophospholipids are absent. Menaquinone 6 is the only respiratory quinone. Homospermidine and 2-hydroxyputrescine are the major polyamines in *Chryseobacterium indologenes*, whereas putrescine and agmatine are minor components (Hamana and Matsuzaki, 1991). The genus' major polyamine is sym-homospermidine (Kämpfer *et al.,* 2009b).

The type species is *Chryseobacterium gleum* comb. nov. The DNA base composition ranges from 33 to 38 mol% G+C. *Chryseobacterium* species are widely distributed in soil, water and clinical sources.

2.3.3. *Ecology*

In reflection to the whole *Flavobacteriaceae* family, *Chryseobacterium* as well as *Elizabethkingia* strains are widely distributed in nature's various ecological niches as summarised earlier in Table 2.3. They are found in water, soil, clinical and food sources (Vandamme *et al.,* 1994b; Jooste and Hugo, 1999; Hugo *et al.,* 2003; de Beer *et al.,* 2005a; Bernardet *et al.,* 2006). Environmental strains may occur in both fresh water and sea water since they exhibit various degrees of halotolerance to sodium chloride (Bernardet *et al.,* 2002; 2006; 2011). Several *Chryseobacterium* strains including closely related species "*Haloanella gallinarum*" occur in sink drains (Bruun *et al.,* 1989; McBain *et al.,* 2003).

The significance of the chryseobacteria in clinical, food and industrial sources, with special emphasis on the two last mentioned sources, will be discussed in the forthcoming sections.

Table 2.4. Differentiating characteristics of the genus *Chryseobacterium* and allied bacteria[a].

Characteristic	***Flavobacterium***	***Chryseobacterium***	***Empedobacter***	***Weeksella***	***Bergeyella***	***Riemerella***
G + C content (mol%)	30-41	33-38	31-33	35-38	35-37	29-35
Respiratory quinone	Menaquinone 6	Menaquinone 6	Menaquinone 6	Menaquinone 6	ND	Menaquinone 6
Habitat	Free living	Free living or parasitic	Free living or parasitic	Parasitic or saprophytic	Parasitic or saprophytic	Parasitic
Pigment production	+ (carotenoid)	+ (flexirubin)[b]	+ (flexirubin)	-	-	-
Saccharolytic metabolism	+	+	+	-	-	+
Capnophilic metabolism	-	-	-	-	-	+
Resistance to penicillin	ND	+	+	-	-	-
DNase activity	V	+[c]	+	-	-	ND
Gelatinase activity	V	+[d]	+	+	+	+[e]
Urease activity	-	V[f]	-	-	+	V[f]
Production of indole	-	V[g]	+	+	+	-
Hydrolysis of esculin	V	+	-	-	-	ND
Growth at 37°C	V	+	V[i]	+	+	+

Growth at 42°C	-	V[f]	-	+	-[j]	+
Growth on MacConkey agar	V	+[k]	+	+	-	-
β-hydroxybutyrate	-	+	+	+	-	ND
Acid production from glucose	+	+[l]	V[i]	-	-	V[f]
Acid production from sucrose	+	-	-	-	-	-

+, Present in all strains; -, absent in all strains; v, variable; ND, not determined
[a] Data were adapted from Vandamme *et al.* (1994a).
[b] Some [*Chryseobacterium*] *meningosepticum* strains are nonpigmented.
[c] Present in all *Chryseobacterium* strains studied except 2 of 12 *Chryseobacterium gleum* strains. Not determined for *Chryseobacterium indoltheticum*.
[d] Present in all *Chryseobacterium* strains studied. Not determined for *Chryseobacterium indoltheticum.*
[e] Most *R. anatipestifer* strains are positive for this characteristic.
[f] Variable within and between species.
[g] Present in all *Chryseobacterium* species except *Chryseobacterium scophthalmum.*
[i] Present in 6 of 7 *E. brevis* strains studied.
[j] Present in 1 of 30 strains studied.
[k] Present in 1 of 30 *Chryseobacterium indologenes* strains studied and in all *Chryseobacterium scophthalmum* strains tested.
[l] Absent in all *Chryseobacterium scophthalmum* strains tested.

2.3.3.1. *Significance in the clinical environment*

Chryseobacterium strains are found in the hospital environment since water is their natural habitat. *Chryseobacterium* and related unidentified bacteria are the most frequently isolated flavobacteria in the clinical laboratory (Holmes and Owen, according to Bernardet *et al.,* 2011). *Chryseobacterium* are not part of the normal flora of humans. They are, however, found on patients' inner and outer body surfaces, where they colonise indwelling devices such as endotracheal tubes and intravascular catheters from where they can spread and occasionally cause bacteraemia, having the potential of colonizing other indwelling devices. (Bernardet *et al.,* 2011).

Chryseobacterium bacteremia was linked to the death of one patient (Kienzle *et al.,* 2000). Cascio *et al.* (2005) reported a case of bacteraemia caused by *Chryseobacterium indologenes* in a diabetic child. *Chryseobacterium indologenes* and *C. gleum* may cause nosocomial infections in humans, usually in neonates or immunocompromised patients (Bernardet *et al.,* 2006). Even though human infections are few, they are potentially serious due to the fact that the patients are immunocompromised and strains have a high frequency of multiple resistance to many antimicrobial agents (Block *et al.,* 1997; Hoque *et al.,* 2001; Michel *et al.,* 2005; Bernardet and Nakagawa, 2006). A study in the department of Food Science at the University of the Free State (UFS) concluded that *C*. *indologenes*, *C*. *indoltheticum* and *C*. *joostei* were the most resistant while *C*. *balustinum* was the most susceptible of the *Chryseobacterium* isolates studied (Van Wyk, 2008).

In animals, *C*. *indologenes* was found to be a pathogen of the leopard frog (*Rana pipiens*; Olson *et al.,* 1992) and bullfrogs (*Rana castesbeiana*; Mauel *et al.,* 2002). *Chryseobacterium balustinum* and *C. scophthalmum* were isolated from diseased fish. The fish pathogens produce extracellular proteases that degrade collagen, elastin, fibrinogen and keratin. Proteases and complex polysaccharide degrading enzymes are responsible for necrotic lesions that occur in infected fish (Bernardet and Nakagawa, 2006).

Pavlov *et al.* (2004) reported that *Chryseobacterium* species were among the most common potentially pathogenic bacteria isolated from samples of treated and untreated water in South Africa.

2.3.3.2. *Significance in the food environment*

Chryseobacterium species are frequently isolated from food environments. Studies in the past by many researchers globally, but also in the Dept of Food Science, UFS, have indicated that *Chryseobacterium* spp. are widely distributed in food sources such as milk, fish, meat and poultry (Hugo *et al.,* 2003; de Beer *et al.,* 2005; 2006). The significance of chryseobacteria in the food environment is regarded to be associated with food spoilage. Changes in the classification of the *Flavobacteriaceae* led to limitations in literature pertaining to food spoilage due to specific members of this family and the recently discovered genera such as *Chryseobacterium* are no exception since they were not considered separately in earlier studies. Consequently, literature on food spoilage in earlier studies generally refers to psychrotrophic bacteria of the flavobacteria group, e.g. as flavobacteria or *Flavobacterium* or CDC group IIb organisms. Literature referring to *Flavobacterium* included many of the currently known *Chryseobacterium* species (de Beer *et al.*, 2005b; Bernardet *et al.,* 2006; 2011).

Food spoilage is a huge economical problem worldwide since a wide variety of microorganisms may initially be present on food and grow if favourable conditions are present. On the basis of susceptibility to spoilage, foods may be classified as non-perishable (or stable), semi-perishable and perishable. The classification depends on the intrinsic factors such as water activity, pH and presence of natural antimicrobial agents. Flour is a non-perishable food because of the low water activity. Apples are semi-perishable since poor handling and poor storage can result in fungal rot. Raw meat is perishable since the intrinsic factors of pH and water activity favour microbial growth (Forsythe, 2000).

The role of *Pseudomonas* and flavobacteria in food spoilage has been well-documented (Garbutt, 1997; García-López *et al.,* 1998, 1999; Forsythe, 2000). While food poisoning is caused by the ingestion of food which exceeds the

infection dose by the consumer who is unaware of the potential problem because the food looks, smells and tastes normal, food spoilage is the culmination of changes that render the food unacceptable to the consumer. It may be due to insect damage, physical injury (caused by bruising, pressure, freezing, drying and radiation), and the activity of the indigenous enzymes in animal and plant tissues. It is also caused by chemical changes not induced by microbial or naturally occurring enzymes and bacteria and fungi activity. The shelf-life of fresh foods is dependant upon the growth and metabolism of the spoilage flora leading to possible changes in pH, formation of toxic compounds, off-odours, gas and slime formation. Lipids and pigments in fat containing foods may be oxidised resulting in undesirable off-flavours and formation of compounds with adverse biological effects or discolouration (Forsythe, 2000). Food spoilage has resulted in economic losses to both the consumer and the industry as well as possible detrimental health effects to the consumers (Garbutt, 1997).

The occurrence and significance of chryseobacteria in specific foods where they occur most commonly will now be discussed in more detail.

a.) *Poultry*

The initial microflora on poultry is a complex mixture similar to that also found on beef, lamb and pork. The organisms originate from the skin, mucous membranes and environmental sources such as soil and water (Molin, 2000). The principal genera on cut-up poultry include *Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Psychrobacter*, *Moraxella*, coryneforms, *Enterobacteriaceae*, *Staphylococcus*, *Micrococcus* (Molin, 2000); *Achromobacter*, *Proteus*, *Bacillus*, *Eberthella*, *Salmonella*, *Alcaligenes*, *Sarcina*, *Streptococcus*, *Escherichia*, *Aerobacter*, *Streptomyces*, *Penicillium*, *Oospora*, *Cryptococcus* and *Rhodotorula* (Mountney and Gould, 1998).

Flavobacteria have been frequently isolated from poultry and meat products but they have seldom been accurately identified (García-López *et al.,* 1998). The incidence of flavobacteria in poultry was reported to be much higher than on fresh meat (Nychas and Drosinos, 1999) while a study by Mai and Conner (2001) on

chicken carcasses found that *Pseudomonas* and flavobacteria had an incidence of 17% and 16% respectively. Poultry skin can carry a range of spoilage organisms (Forsythe, 2000) and spoilage is generally restricted to the outer surfaces of the skin and cuts (Banwart, 1989). The spoilage is due to the degradation of proteins producing volatile off-flavours such as indole, dimethyl-disulphide and ammonia. Chemical oxidation of unsaturated lipids results in a rancid off-flavour (Forsythe, 2000). Off-flavours appear at a bacterial load ranging between 6 and 8 log cfu cm^{-2} (Banwart, 1989), followed shortly thereafter by occurrence of sliminess at 8 log cfu cm^{-2} (Jay, 1992) and then the various types of discolourations (Jackson *et al.,* 1997).

Within the animal's body, the meat itself is sterile but it can become easily contaminated during slaughter, abattoir practice, handling during processing and improper storage (Forsythe, 2000). Commercial broiler processing includes among other operations, scalding, defeathering, evisceration and chilling. These steps reduce microbial contamination of broiler carcasses (Geornaras and von Holy, 2000). However, cross contamination between carcasses, processing water and equipment may actually increase the level of carcass contamination (Thomas and McMeekin, 1980; Fries and Graw, 1999). *Chryseobacterium* strains form part of the microflora that colonise the feathers and skin of live broilers. Psychrotolerant organisms that survive processing may multiply during refrigerated storage and cause spoilage of fresh poultry (Russel *et al.,* 1996; Hinton *et al.,* 2002; Hinton *et al.,* 2004).

Previous studies in the department of Food Science, UFS, found that *Chryseobacterium* species were present throughout the processing unit of a poultry processing plant. Environmental sources such as dust, most likely contributed to contamination levels of psychrotolerant, yellow pigmented colonies especially *Chryseobacterium* on raw chicken meat. *Chryseobacterium* strains accounted for 8.3% of the total bacterial count on washed whole birds despite lowered total contamination by spoilage organisms (de Beer, 2005).

b.) *Fish*

Flavobacteria occur regularly on fish (Jooste and Hugo, 1999; de Beer *et al.,* 2005) among other heterogeneous flora such as *Acinetobacter*, coryneforms, *Cytophaga*, *Micrococcus*, *Psychrobacter* (former "*Moraxella*-like), *Aeromonas*, *Shewanella* and *Pseudomonas* (Molin, 2000) but their role in spoilage is minor compared to that of *Pseudomonas* (McMeekin, 1982). The initial numbers of bacteria on fish are normally higher than on mammalian meat (Molin, 2000). Fish habour 10^3 – 10^5 cfu cm^{-2} on the skin, 10^3 – 10^4 cfu g^{-1} on the gills, up to 10^9 cfu g^{-1} in the intestines, and often close to 10^5 cfu cm^{-2} after filleting (Molin, 2000).

Microorganisms play a major role in organoleptic deterioration of fish even though autolytic processes are responsible for many catabolic biochemical reactions in fresh fish (Leisner and Gram, 1999). Catabolites produced by fish spoilage organisms are similar to those produced by organisms during spoilage of poultry and red meat. However, one metabolite, trimethylamine, is formed during anaerobic respiration of trimethylamine oxide which is found in abundance in raw fish (Leisner and Gram, 1999). Trimethylamine has an ammonia-like "fishy" odour (Gram and Dalgaard, 2002). Gram-negative bacteria like *Pseudomonas* species and flavobacteria also produce typical fish spoilage odours described as fruity, pungent and musty (Engelbrecht *et al.,* 1996). In an earlier study, Engelbrecht (1992) found that *Enterobacteriaceae*, *Aeromonas*/*Vibrio, Pseudomonas*, *Flavobacterium* and *Alcaligenes* might frequently be regarded as active spoilers of chilled Cape fish species on the basis of off-odour production, proteolysis and hydrogen sulphide production. The off-odour is due to the production of volatile esters like ethyl acetate and volatile sulphide compounds such as methylmercaptan and dimethylsulphide (Forsythe, 2000).

c.) *Milk and milk products*

In the dairy environment, *Flavobacterium* were first isolated from milk and butter during a research project in the Department of Food Science at the University of the Free State in South Africa (Jooste, 1985; Hugo *et al.,* 2003). The significance of *Chryseobacterium* strains in the dairy environment indicates a role in spoilage of milk and milk products due to their ability to produce very heat-stable metalloproteases with an affinity for casein (Venter *et al.,* 1999). They are also

linked to the presence of off-flavours in milk and milk products, changes in milk as a substrate for starter cultures and reduced cheese yield.

It was also found that the practical importance of dairy flavobacteria lies as much in their psychrotolerant growth and consequent proteinase production in refrigerated milk as in their contamination of the milk via poorly sanitized pipelines and equipment (Jooste *et al*., 1986). In subsequent studies, Hugo and Jooste (1997) and Hugo *et al*. (1999), isolated *Chryseobacterium indologenes*, *C. gleum*, CDC group IIb, and the then new species *C. joostei* from milk. *Chryseobacterium* were also isolated from a lactic acid beverage in Japan where *Chryseobacterium shigense* has been considered as a part of the normal flora of the lactic acid beverage (Shimumora *et al.*, 2005). Recent research in the Department of Evolutionary Microbiology at the University of Haifa in Israel, on the diversity of culturable psychrotolerant bacteria in raw cow's milk isolated *Chryseobacterium* strains which led to the description of the novel species *Chryseobacterium haifense*, *C. bovis* and *C. oranimense* (Hantsis-Zacharov and Halpern, 2007; Hantsis-Zacharov *et al*., 2008a; 2008b).

d.) *Red meat*

Red meat is highly perishable. Contaminating genera that have been isolated include *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Flavobacterium*, *Moraxella*, *Proteus*, *Pseudomonas*, *Shewanella*, *Salmonella*, *Yersinia*, *Bacillus*, *Brochothrix*, *Clostridium*, *Corynebacterium*, *Lactobacillus*, *Listeria*, *Enterococcus*, *Lactococcus*, *Micrococcus*, *Pediococcus*, *Staphylococcus*, *Mucor*, *Rhizopus*, *Thamnidium*, *Cladosporium*, *Geotrichum*, *Sporotrichum* and *Candida* (Enfors *et al.,* 1979; Blikstad *et al.,* 1981; Erichsen and Molin, 1981; Garbutt, 1997). The microbial diversity on newly-cut beef is very high but the number of bacterial cells is very low, ranging between 100–1 000 cfu cm^{-2} (Blickstad *et al.,* 1981; Blickstad and Molin, 1983; Jackson *et al.,* 1992; Olofsson *et al.,* 2007).

Despite the numerous and varied types of meat contaminating microflora, not all the contaminants are involved in the spoilage process since it is always

dominated by a few and sometimes only one organism (Garbutt, 1997). Which component of the microflora becomes dominant is determined by a complex interaction between implicit, extrinsic and intrinsic factors (Garbutt, 1997). A study by Olofsson *et al.* (2007) on the composition of the bacterial population of refrigerated beef, identified by 16S rRNA gene analysis and pure culture technique, revealed initial domination by *Bacillus*-like sequences using direct 16S rRNA gene analysis while viable plate count was dominated by *Chryseobacterium* species. Analyses of the multiplying beef populations using both techniques indicated *Pseudomonas* species as the dominating group.

An earlier study by Jay *et al.* (2003) found that Gram-negative strains increased with time and *Pseudomonas* dominated the spoilage microflora of ground beef while Bernardet *et al.* (2005) reported that *Chryseobacterium gleum* and *Chryseobacterium indologenes* are often initially present on raw meat. Similarly, McMeekin (1982) reported that *Flavobacterium* are a constant part of chilled meats and poultry but are unable to compete with *Pseudomonas* during storage. The inhibition of other organisms by *Pseudomonas* appears to be due to their inability to compete with *Pseudomonas* for the available oxygen (Nottingham, 1982).

Spoilage of red meat results in offensive off-odours, possible slime production, discolouration of a specific area and undesirable flavours due to metabolic end products formed. Flavobacteria produce alcohols like methanol and ethanol, sulphur compounds such as dimethylsulphide, methylmercaptan and methanethiol, aldehydes, ketones, esters and amines from amino acid metabolism (Banwart, 1989). The offensive odours can be described as fishy, foul, sulphuric and ammonia-like (Nychas and Drosinos, 1999).

2.3.3.3. *Significance in the industrial environment*

Some strains of *Chryseobacterium* environmental isolates have been shown to degrade various toxic compounds. Even though pentachlorophenol (PCP) is used as a pesticide in wood preservation and as a disinfectant in the food industry, it poses significant health hazards. A mixture of *C. gleum*, *Agrobacterium*

radiobacter and *Pseudomonas* sp. was used in the cleanup of PCP. Degradation rates for PCP were lower for the individual isolates compared to that observed for the mixed culture but *C. gleum* showed the highest individual PCP degradative ability (Yu and Ward, 1996).

Another mixture of *C. indologenes* SB1, *Comamonas testosteroni* SB2, *Pseudomonas corrugata* SB4 and *Stenotrophomonas maltophilia* SB5 was found to degrade aniline and 4-chloroaniline in contaminated agricultural soil in Indonesia (Radianingtyas *et al.,* 2003). A strain of *C. indologenes* isolated among soil bacteria in Spain was able to degrade toxic furan and phenolic compounds emanating from the acid treatment of lignocellulose (Lopez *et al.,* 2004).

A potential application of *Chryseobacterium* was shown by a novel enzyme from "*C. proteolyticum*." It was shown to deamidate glutaminyl residues in proteins (Yamaguchi *et al*., 2001). Protein deamidation is regarded as a promising method to improve protein functionality such as emulsifiers or foaming agents in food systems (Hamada, 1994).

Another potential application was demonstrated by a strain of *Chryseobacterium* which was shown to secrete a novel endopeptidase capable of cleaving the human plasminogen and could consequently reduce bacterial invasion (Lijnen *et al*., 2000).

Although the chryseobacteria are mainly regarded as food spoilers, the broader significance of *Chryseobacterium* species in the food industry has long been debated. Recently, however, strains of *Chryseobacterium* have been isolated from poultry feather waste. These strains were able to break down the insoluble keratin in the feathers by the production of keratinases (Riffel *et al.,* 2003; Casarin *et al.,* 2008). Keratinolytic enzymes may have important uses in biotechnological processes aimed at decreasing the pollution of the environment (Shih, 1993; Onifade *et al.,* 1998). Their application has also been extended to detergent and leather industries where they serve as speciality enzymes. Besides, they also find application in wool and silk cleaning. In the leather industry, better dehairing potential of these enzymes has led to the development

of greener hair-shaving technology and personal care products. Further, they show great potential in the new area of prion degradation for treatment of the "mad cow" disease (Langeveld *et al.,* 2003; Gupta and Ramnani, 2006).

Feathers are produced in large amounts as a waste by-product at poultry processing plants (Williams *et al.,* 1991; Riffel *et al.,* 2003). They represent a problem for the industry since they pollute the environment. Their utilization is difficult because although the feathers consist mainly (90%) of protein, this protein is keratin (Sangalli and Brandelli, 2000), which is chemically virtually inert. Keratins are fibrous proteins that make up a variety of organic materials found in living organisms. They form a major component of the hard but non-mineralized structures found in the epidermis and in its appendages namely; feathers, hair, nails, horns, hoofs, scales, wool, beaks, claws and shells (Gupta and Ramnani, 2006). All proteins are created through various combinations of the same 20 amino acids but the most prevalent amino acids found in keratins are glycine, alanine and cysteine.

Keratin molecules are helical (Figure 2.5) and they twist around each other in a parallel manner to form micro- and macro-fibrils that warrant stability to the fibre (Kreplak *et al.,* 2004; Zerdani *et al.,* 2004). Intermolecular hydrogen bonds increase the strength of the keratin while formation of many covalent bonds called, disulphide bridges, between the sulphur atoms on two cysteines on separate polypeptide chains allows for cross-linkage of these chains. This results in a very rigid protein structure in the raw feathers that renders them insoluble with a very low digestibility of 5%.

Keratins are classified into α- and β-keratins on the basis of their secondary structure conformation. Alpha-keratins are found in mammals only and are responsible for the make up of hair, nails, horns, hooves and claws. Beta keratins are found in birds, reptiles and amphibians forming feathers, claws, nails, scales, shells and beaks. Beta-keratins are harder compared to α-keratins (Voet and Voet, 1995; Gupta and Ramnani, 2006).

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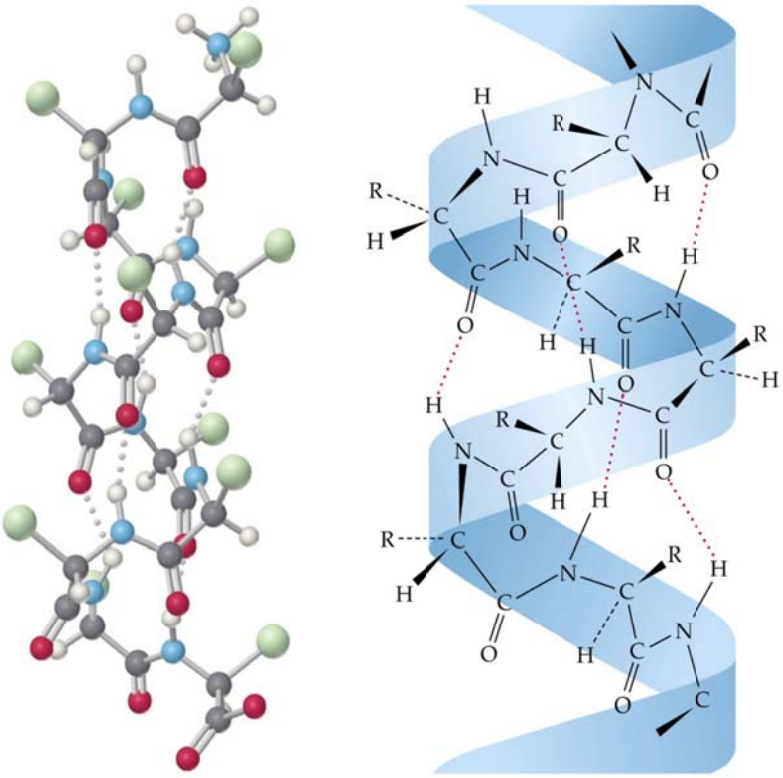

Fig. 2.5. Intermolecular hydrogen bonding in keratin which results in increased strength of the protein (http://www.itech.dickinson.edu/chemistry/?cat=69).

The intensive cross-linkages in keratins hinder their degradation by commonly known proteolytic enzymes like trypsin, pepsin and papain (Papadopoulos, 1986; Sangalli and Brandelli, 2000). However, they are efficiently degraded by keratinases from bacteria, actinomycetes and fungi (Onifade *et al.,* 1998). Keratinolytic enzymes are produced by some species of *Bacillus* such as *Bacillus licheniformis* PWD1 (Williams *et al.,* 1990), *Bacillus* sp. P-001A (Atalo and Gashe, 1993), the fungus *Aspergillus fumigatus* (Santos *et al.,* 1996) and the actinomycete *Streptomyces pactum* DSM 40530 (Bockle *et al.,* 1995). A comprehensive list of organisms that produce keratinolytic enzymes and the time required for complete keratinolysis is shown in Table 2.5.

The most keratinolytic group among fungi belongs to *fungi imperfectii* including the following genera: *Chrysosporium*, *Aspergillus*, *Alternaria*, *Trichurus*,

Curvularia, *Cladosporium*, *Fusarium*, *Geomyces*, *Gleomastis*, *Monodictys*, *Myrothecium*, *Paecilomyces*, *Stachybotrys*, *Urocladium*, *Scopulariopsis*, *Sepedonium*, *Penicillium* and *Doratomyces*. However, they do not have much commercial value as most of them are categorized as dermatophytes (Gradisar *et al.,* 2000). Even though keratinolytic activity is mostly confined to Gram-positive organisms, the recently isolated Gram-negative *Chryseobacterium* sp. kr6 is comparable to Gram-positive organisms (Table 2.5) and can, like *Bacillus subtilis*, equally completely degrade feather keratin in 72 hours with optimum keratinase production at 48 hours (Kim *et al.,* 2001; Riffel *et al.,* 2003).

Keratinases' exact nature and uniqueness for keratinolysis is not fully understood (Gupta and Ramnani, 2006). Ramnani and Gupta (2007) found that extracellular proteases cooperatively act with intracellular disulphide reductases to bring about keratinolysis. The reductases help in breakage of disulphide bonds in the keratin thereby allowing proteases to access their peptide bond substrates (Bockle and Muller, 1997). Cell free enzyme is incapable of complete feather degradation since it requires a continuous supply of reductant in the form of live bacterial cells (Ramnani and Gupta, 2007).

The competition between humans and livestock for quality sources of protein is likely to increase continuously because of the ever-increasing world population. The effort to find viable alternative protein sources for livestock is, therefore, important for improved livestock production in the future. Hydrolyzed feather meal has been of interest in nutrition research because of its high protein content. It is more digestible and does not suffer from the disadvantages of anti-nutritional factors, such as tannins, glucosinolates, lectins and trypsin inhibiting factors found in high protein plant sources such as legumes (AFMA, 2008). This protein, however, is deficient in essential amino acids such as lysine, methionine, histidine and tryptophan (FAO, 2008a). The hydrolysed feathers can also be converted to fertilizers, glues and edible films or used for the production of rare amino acids such as serine, cysteine and proline (Papadopolous *et al.,* 1986; Dalev and Neitchev 1991; Choi and Nelson, 1996; Yamauchi *et al.,* 1996).

Table 2.5. Keratinolytic organisms, keratinase production temperature and time for complete keratinolysis

Organism	Type	Aerobic/ Anaerobic	Production Temp °C	Time taken for complete keratin degradation/keratinolysis	Reference
Bacillus licheniformis PWD1	Bacterium	Aerobic/ facultatively anaerobic	50	10 days	Williams *et al*., 1990
Bacillus licheniformis K-508	Bacterium	Aerobic/ facultatively anaerobic	45	4 days	Manczinger *et al*., 2003
Fervidobacterium pennavorans	Bacterium	Anaerobic	70	48 hrs	Friedrich and Antranikian, 1996
Kocuria rosea	Bacterium	Aerobic	40	55% within 96 hrs	Vidal *et al*., 2000
Bacillus subtilis KS1	Bacterium	Aerobic/ facultatively anaerobic	40	72 hrs	Kim *et al*., 2001
Bacillus pumilus	Bacterium	Aerobic/ facultatively anaerobic	40	Data not available	Pissuwan and Suntornsuk, 2001
Bacillus cereus	Bacterium	Aerobic/ facultatively anaerobic	30	Data not available	Pissuwan and Suntornsuk, 2001
Bacillus sp. FK28	Bacterium	Aerobic/ facultatively anaerobic	37	Data not available	Pissuwan and Suntornsuk, 2001
Bacillus species P-001A	Bacterium	Aerobic/ facultatively anaerobic	Data not available	Data not available	Atalo and Gashe, 1993
Thermoanaerobacter keratinophilus sp. nov.	Bacterium	Anaerobic	70	70% degradation within 10 days	Rissen and Antranikian, 2001
Xanthomonas maltophila POA-1	Bacterium	Aerobic	30	Data not available	De Toni *et al*., 2002

Fervidobacterium islandicum AW-1	Bacterium	Anaerobic	70	48 hrs	Nam *et al*., 2002
Stenotrophomonas sp. D1	Bacterium	Aerobic	20	2.5 hrs	Yamamura *et al*., 2002
Bacillus pseudofirmis AL-89	Bacterium	Aerobic/ facultatively anaerobic	37	Data not available	Gassesse *et al*., 2003
Chryseobacterium sp. kr6	Bacterium	Aerobic	25-30	72 hrs	Riffel *et al*., 2003
Microbacterium arborescens kr10	Bacterium	Aerobic	30	72 hrs	Thys *et al*., 2004
Aspergillus fumigatus	Fungi	Aerobic	Data not available	Data not available	Santos *et al.,* 1996
Streptomyces pactum DSM 40530	Actinomycete	Aerobic	Data not available	Data not available	Bockle *et al.,* 1995
Microsporum gypseum	Fungi	Aerobic	Data not available	Data not available	Kunert, 1989

Currently, feather meal is hydrolysed mainly by cooking at a high temperature under sufficient pressure. This process, however, requires the input of much energy, which also can have a negative effect on the environment. The poultry feathers can furthermore be hydrolysed with chemicals by cooking in an open kettle with a solution of sodium sulphite in alcohol and water (FAO, 2008b). Eggum (1970) reported that acid hydrolysis (HCl at pH 6 for 20 hours) can also be used to produce a meal of high digestibility and similar biological value. However, the current processes to obtain feather meal are expensive, damaging to the environment and they destroy certain amino acids, yielding products with poor digestibility and variable nutritional quality (Wang and Parsons, 1997). The use of crude keratinases significantly increases the amino acid digestibility of raw feathers and commercial feather meal (Lee *et al.,* 1991).

2.4. Conclusions

The taxonomy of the family *Flavobacteriaceae*, from its status as a genus of the family *Bacteriaceae* in 1923 to a validated family in 1992, was characterized by poor description, uncertain taxonomic status, considerable modifications leading to new restrictions and definitions. The validation of the family by Reichenbach in 1992 clarified its taxonomic rank but the genus *Flavobacterium* remained heterogenous until it was thoroughly emended by Bernardet *et al*. (1996) giving birth to the genus *Chryseobacterium*, together with the genera *Bergeyella* and *Empedobacter*. The genus *Chryseobacterium* in turn gave birth to the genus *Elizabethkingia* in 2005. *Chryseobacterium* species increased phenomenally from the initial six species in 1994 to the current 58, and there are prospects for other new species to be descrided. It can be concluded that the current taxonomic status of *Flavobacteriaceae* and *Chryseobacterium* is homogeneous and valid since both genotypic and phenotypic standards were used.

Original taxonomic methods relied on phenotypic investigations to delineate taxa. However, these proved to be inadequate since, among other reasons, the genotype was not included. The chemotaxonomic approach refined the differentiation of taxa and with the advent of molecular techniques such as PFGE, rRNA sequencing, DNA-DNA hybridization and RFLP, resolution of closely

related genera and species became possible. Taxonomy cannot rely on a single method since characteristics of organisms are not encoded on a single molecule hence, taxonomists adopted a consensus approach to bacterial systematics in the form of polyphasic taxonomy.

Members of the genus *Chryseobacterium* can play a pivotal role in biotechnology and bioremediation since they are able to produce a variety of enzymes including the relatively heat stable keratinases which exhibit great potential in environmentally friendly industrial and technological processes. Degradation of feather keratin by *Chryseobacterium* keratinases can provide an inexpensive way to hydrolyse feathers from the poultry industry releasing important proteins and amino acids that can vastly improve the nutrition of stock and ultimately humans.

CHAPTER 3

THE OCCURRENCE OF *Chryseobacterium* SPECIES IN POULTRY FEATHER WASTE

Abstract

A total of 466 yellow-pigmented bacterial isolates were obtained from chicken feather waste and feather meal samples from three poultry processing plants in South Africa. Two plants (A and B) were in Bloemfontein, the Free State province and one plant (C) was in Johannesburg, Gauteng province. An incubation regime of 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h gave higher total bacteria counts and total yellow colony counts compared to incubation at 4 ^{o}C for 10 days and 25 ^{o}C for 48 h. Eight of the samples that were at different stages of decomposition after burial showed that the yellow-pigmented species were unable or barely able to survive longer than one week after burial. The BIOLOG Gen II microbial identification system identified seven out of 35 isolates. Four isolates (1_F178, 29_FM14, 7_F195, 25_F82) belonged to the family *Flavobacteriaceae* while the other three were identified as species of the genus *Burkholderia*. Twelve presumptive *Chryseobacterium* isolates, five each from poultry processing plants A and B; and two from plant C, were selected for further taxonomic studies.

3.1. Introduction

The genus *Chryseobacterium* emerged from the ruins of the erstwhile *Flavobacterium* genus which had lumped together predominantly yellow-pigmented species that were not related according to modern genotypic standards (Vandamme *et al*., 1994a). Yellow pigmented bacterial species (including members of the present genus *Chryseobacterium*) previously included in the *Flavobacterium* genus, were assigned to new genera within the larger *Flavobacteriaceae* family. Currently, the genus *Chryseobacterium* typically consists of Gram-negative, oxidase positive, non-endospore-forming, non-motile and yellow-pigmented rods (Bernardet *et al*., 2011). *Chryseobacterium* species inhabit a variety of environments such as activated sludge (Kämpfer *et al*., 2003), beer bottling plants (Herzog *et al*., 2008), soil (Weon *et al*., 2008) and food (Hugo *et al*., 2003).

In the food environment, red meat and poultry are key sources of protein and their spoilage characteristics are very similar. The main difference is that poultry is more perishable since it has a slightly higher pH (Mossel *et al*., 1995). In the processing of poultry, the birds are the main vehicles of transmission of contaminating organisms such as *Salmonella*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Pseudomonas* and flavobacteria, and these are found on the feathers, skins and in intestines (Mead, 1989). The scalding process at about 52 ^{o}C, has minimal lethal effect. Scalding is followed by plucking and the rubber fingers of the plucking machines facilitate cross-contamination between feathers and carcasses (Mossel *et al*., 1995).

Feather waste is currently used to a limited extent as a dietary protein supplement for animal feed, but the poultry industry generates huge amounts for which effective disposal is challenging from an environmental and economic perspective (Kelleher *et al*., 2002; Riffel *et al*., 2003). Feather disposal is particularly difficult since they are made up of keratin which is insoluble and resistant to attack by most proteases (Riffel *et al*., 2003). Several methods have been used to dispose of the waste and these include burial, burning, natural gas production and treatment for animal feed. Most of the feathers are currently disposed of by burial or incineration. These methods are expensive and contaminate the air, soil and water. Feathers, however, do not accumulate in nature since keratin can be degraded by some microorganisms such as *Bacillus licheniformis* (Ramnani *et al*., 2005), *Burkholderia*, *Pseudomonas* and *Microbacterium* species (Brandelli and Riffel, 2006). Keratinolytic *Chryseobacterium* species have also been isolated from poultry feather waste (Riffel *et al*., 2003; Brandelli, 2005). *Chryseobacterium* species kr6 had the highest activity (Riffel and Brandelli, 2006).

Phenotypic identification of feather waste microbiota can be accomplished using the BIOLOG Gen II microbial identification system. It is a tool that employs the organism's ability to utilize specific carbon sources in 96-well microplates with 95 substrates to produce a characteristic pattern or metabolic fingerprint. This is then compared to a BIOLOG data base.

The first aim of this study was to isolate yellow-pigmented isolates from chicken feather waste and feather meal samples from poultry processing plants and to screen for *Chryseobacterium* species. The second aim was to perform a preliminary phenotypic identification using the BIOLOG Gen II microbial identification system.

3.2. Materials and Methods

3.2.1. *Samples collected*

Twenty-one feather waste samples were collected from three poultry processing plants; two plants in Bloemfontein, the Free State province (designated A and B) and the third plant in Johannesburg, Gauteng province, South Africa (designated C). Three of the samples were collected from plant A, 16 from plant B, and two from plant C. Since most of the feather waste was disposed of by burying, eight of the 16 samples from plant B were collected from the abattoir as follows: Freshly plucked, before scalding process; freshly plucked after scalding; after scalding but before burial; after burial for 1 week; 3, 5, 12, and 15 months. Eight chicken feather meal samples were also obtained from plant C.

3.2.2. *Isolation of Chryseobacterium species from feather waste and feather meal samples*

3.2.2.1. *Total bacterial counts and total yellow-pigmented colony counts*

Ten grams of feather waste or feather meal were added to 90 ml buffered peptone water (BPW; Merck 63725) and mixed vigorously. Serial dilutions were made in BPW and pour-plated in duplicate using Nutrient Agar (Oxoid CM3) and/or Standard Plate Count Agar (SPCA; Oxoid CM463). Incubation was as shown in Table 3.1.

Yellow-pigmented colonies were enumerated and streaked out on respective Nutrient Agar or SPCA plates until pure colonies were obtained. The isolates

were maintained as freeze-dried cultures on filter paper discs according to Britz and Kriel (1973) and stored in sealed Petri dishes at -20 ^{o}C. For shorter term maintenance, they were cultured on Nutrient Agar slants, stored at 4 ^{o}C and re-streaked every four to six weeks.

Table 3.1. Media and incubation conditions used in making bacterial isolates from feather waste and feather meal samples.

Plant	Media	Number of samples	Incubation temperature and time
A	SPCA and NA	3	i. 4 ^{o}C for 10 days ii. 4 ^{o}C for 48 h then 25 ^{o}C for 48 h iii. 25 ^{o}C for 48 h*
B	SPCA	8	i. 4 ^{o}C for 10 days ii. 4 ^{o}C for 48 h then 25 ^{o}C for 48 h iii. 25 ^{o}C for 48 h*
B	SPCA	8	4 ^{o}C for 48 h then 25 ^{o}C for 48 h
C	SPCA	10	4 ^{o}C for 48 h then 25 ^{o}C for 48 h

*, Hugo and Jooste (2003); SPCA, Standard Plate Count Agar; NA, Nutrient Agar.

3.2.2.2. *Preliminary screening regime for Chryseobacterium species*

A total of 466 yellow-pigmented isolates were subjected to a battery of tests to screen for *Chryseobacterium* isolates based on the characteristics for differentiating *Chryseobacterium* species (Bernardet and Nakagawa, 2006). The methods employed were according to MacFaddin (1980) unless otherwise stated. The tests used to screen for *Chryseobacterium isolates* and the reactions obtained are shown in Table 3.2. The seven reference strains used are shown in Table 3.3.

Table 3.2. Tests and reactions used to screen for *Chryseobacterium* isolates.

Test	Reaction
Colony pigment	Yellow, orange-yellow
Flexirubin pigment type	Positive
Gram stain	Negative
Cell morphology	Small rods (coccobacilli-like under 100X magnification oil immersion light microscope)
Oxidase production	Positive
Catalase production	Positive
Motility	Negative
Acid production from glucose	Positive[a]
DNase production	Positive
Indole production (Hugo, 1997)	Positive[a]
Gelatine degradation	Positive
Casein hydrolysis	Positive

[a], Positive for all *Chryseobacterium* species except *C. scophthalmum*

Table 3.3. *Chryseobacterium* reference strains used in this study.

Reference organism	Culture collection number
Chryseobacterium gleum	NCTC 11432^T
C. joostei	ATCC 18212^T
C. balustinum	NCTC 11212^T
C. indologenes	LMG 8329^T
C. scophthalmum	LMG 13028^T
C. vrystaatense	LMG 22846^T
C. bovis	LMG 24227^T

3.3. Results and discussion

3.3.1. *Isolation of yellow-pigmented species from feather waste samples from processing plant A*

The three samples from poultry processing plant A were used to evaluate optimum media and incubation conditions. Counts were made of all yellow and orange-yellow colonies (TYCC) as well as the total bacterial colonies (TBC) and their mean counts are shown in Figure 3.1. The mean TYCC for all incubation conditions ranged from 2 log cfu g^{-1} to 7 log cfu g^{-1}. For both Nutrient Agar (NA) and Standard Plate Count Agar (SPCA), the highest number of yellow-pigmented colonies was obtained after incubation at 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h. The initial low temperature suppressed the growth of fast-growing mesophiles while giving a competitive advantage to the slow-growing psychrotolerant species. Standard Plate Count Agar gave a higher mean of 6 log cfu g^{-1} compared to Nutrient Agar which gave a mean TYCC of 5 log cfu g^{-1}.

The least TYCC for both media were obtained after incubation at 25 ^{o}C for 48 h. This was possibly due to slow growth by the yellow-pigmented strains such as *Chryseobacterium* species that have a longer lag phase. On the other hand, mean TYCC counts after incubation at 4 ^{o}C for 10 days were higher in both media compared to incubation at 25 ^{o}C for 48 h. The reason for this may be that the yellow-pigmented strains could have been psychrotolerant or psychrophilic and were able to survive better. However, they were dominated by other psychrophiles as shown by the high mean TBCs.

Feathers are one of the potential sources of contamination of chicken meat during processing. Several studies reported the dominance of *Pseudomonas* and other species on chicken meat, beef and poultry. Mai and Conner (2001) reported an incidence of *Pseudomonas* and *Flavobacterium* on chicken meat of 17 and 16% respectively. Jay *et al*. (2003) reported that most organisms do not successfully compete with *Pseudomonas* under refrigerated conditions. A study by Belak *et al*. (2011) found that *Pseudomonas* species dominated the microbiota

of chicken meat stored at chill temperatures. The TBC across all incubation regimes remained fairly constant at about 7 log cfu g^{-1}.

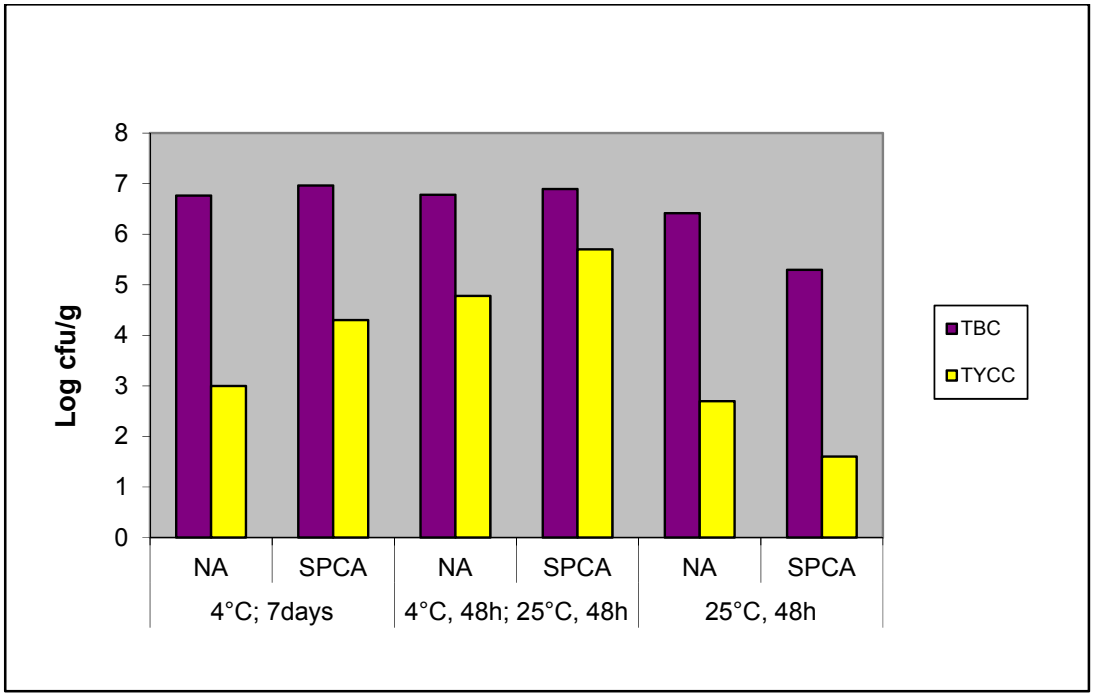

Fig. 3.1. Evaluation of media and incubation regimes for isolation of *Chryseobacterium* species from feather waste samples from plant A.

3.3.2. *Isolation of yellow-pigmented species from plant B's feather waste disposal process and plant C's feather meal samples*

Figures 3.2, 3.3 and 3.4 show the mean TBC and TYCC on SPCA incubated at 4 ^{o}C for 10 days; 4 ^{o}C for 48 h then 25 ^{o}C for 48 h; and 25 ^{o}C for 48 h respectively. Three of the eight samples yielded yellow colonies (Figure 3.2). These samples consisted of feathers that were freshly plucked before the scalding process (FBS), freshly plucked but after the scalding process (FAS), and feathers after scalding but before burial (ASBB) with counts being 2, 3 and 4 log cfu g^{-1} respectively.

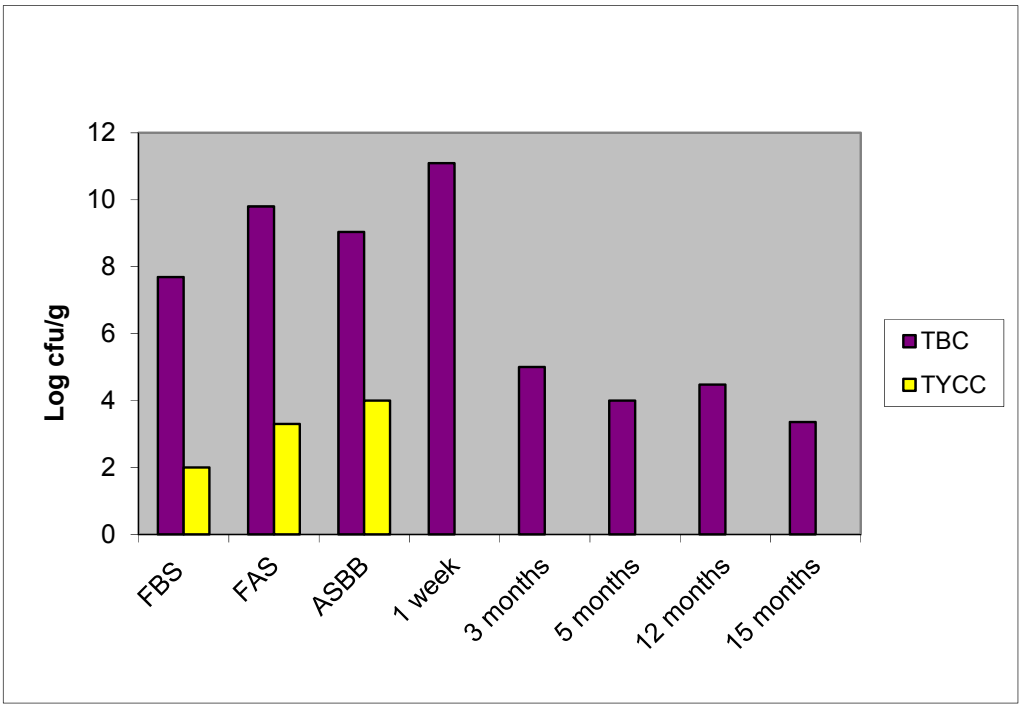

Fig. 3.2. Bacterial counts of feather waste samples from plant B buried from 0 to 15 months and incubated at 4 ^{o}C for 7 days. FBS, Freshly plucked, before scalding process; FAS, Freshly plucked, after scalding process; ASBB, after scalding, before burial.

Higher colony counts after the scalding process may have been caused by post-process contamination from rubber fingers of the plucking machines, work surfaces or equipment (Mossel *et al*., 1995). Non-yellow colony counts increased from the day of slaughter up to one week after burial of the feather waste and then decreased to a mean of 4 log cfu g^{-1} after three to 15 months.

Incubation at 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h yielded the highest TYCC after one week of burial at 11 log cfu g^{-1} followed by ASBB counts at 9 log cfu g^{-1} (Figure 3.3). Yellow colonies were not found in the FAS sample possibly due to the sensitivity of these organisms to heat and that there was no re-contamination immediately after the scalding process. Yellow colonies were not found after 3, 5 and 15 months. This may have been due to a build up of heat below the surface leading to the death of heat sensitive strains. However, it was possible that the

strains found after burial for 12 months at a count of 6 log cfu g^{-1} were heat resistant.

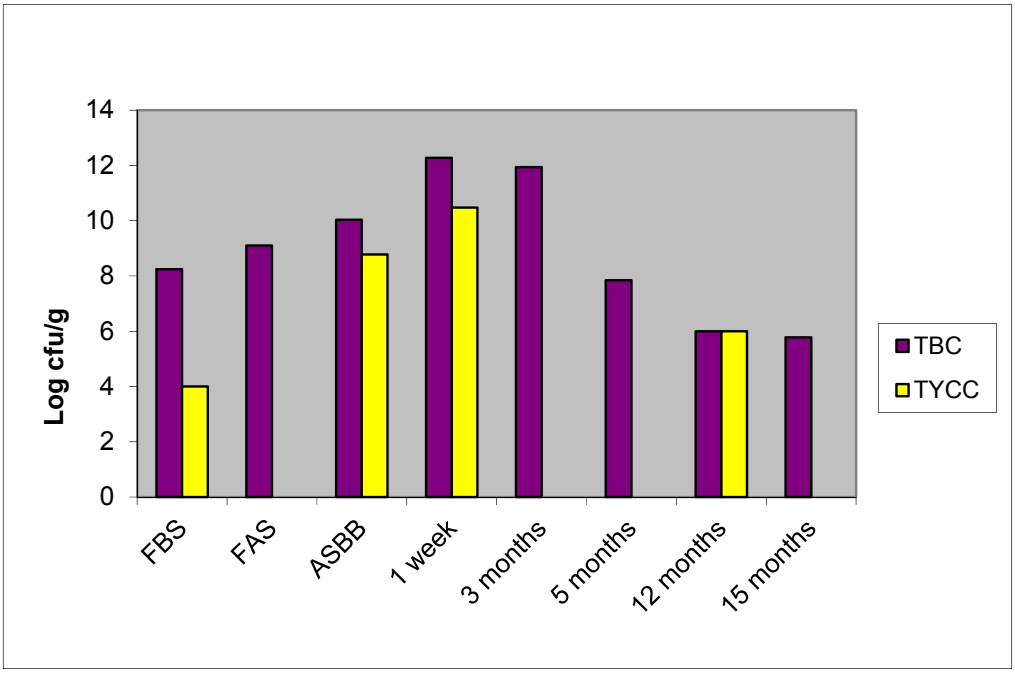

Fig. 3.3. Bacterial counts of feather waste samples from plant B buried from 0 to 15 months and incubated at 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h. FBS, Freshly plucked, before scalding process; FAS, Freshly plucked, after scalding process; ASBB, after scalding, before burial.

Only the ASBB sample gave a TYCC of 9 log cfu g^{-1} after incubation at 25 ^{o}C for 48 h (Figure 3.4). This may be a result of domination of the yellow-pigmented strains by other non- yellow-pigmented species. A comparison of the three incubation regimes revealed that the highest TYCC from mixed and competing populations were obtained after incubation at 4 ^{o}C for 48 h then 25 ^{o}C for 48 h, followed by 4 ^{o}C for 7 days and the least was incubation at 25 ^{o}C for 48 h.

An overview of all the buried samples indicated that the yellow-pigmented strains (which include *Chryseobacterium*) did not survive well after being buried for more than one week. This may be because the feather waste was buried about one

metre below the surface and the yellow-pigmented organisms did not survive in the high compost-like temperatures that were generated.

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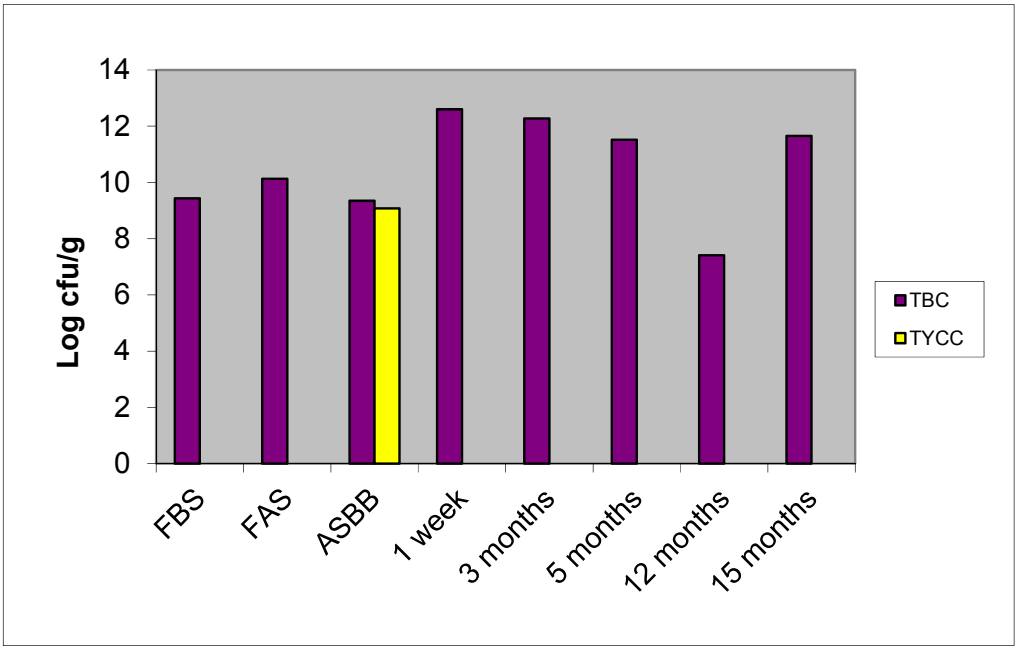


Fig. 3.4. Bacterial counts of feather waste samples from plant B buried from 0 to 15 months and incubated at 25 ^{o}C for 48 h. FBS, Freshly plucked, before scalding process; FAS, Freshly plucked, after scalding process; ASBB, after scalding, before burial.

An incubation regime of 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h was chosen for subsequent isolations. Eight feather waste samples from processing plant B gave TBC ranging from 3.2 to 4.9 log cfu g^{-1} while the TYCC range was 1.3 to 3.7 log cfu g^{-1} (Figure 3.5). The other eight feather meal samples from processing plant C gave TBC ranging from 6.2 to 9 log cfu g^{-1} while the TYCC range was 1.5 to 6.2 log cfu g^{-1} (Figure 3.6).

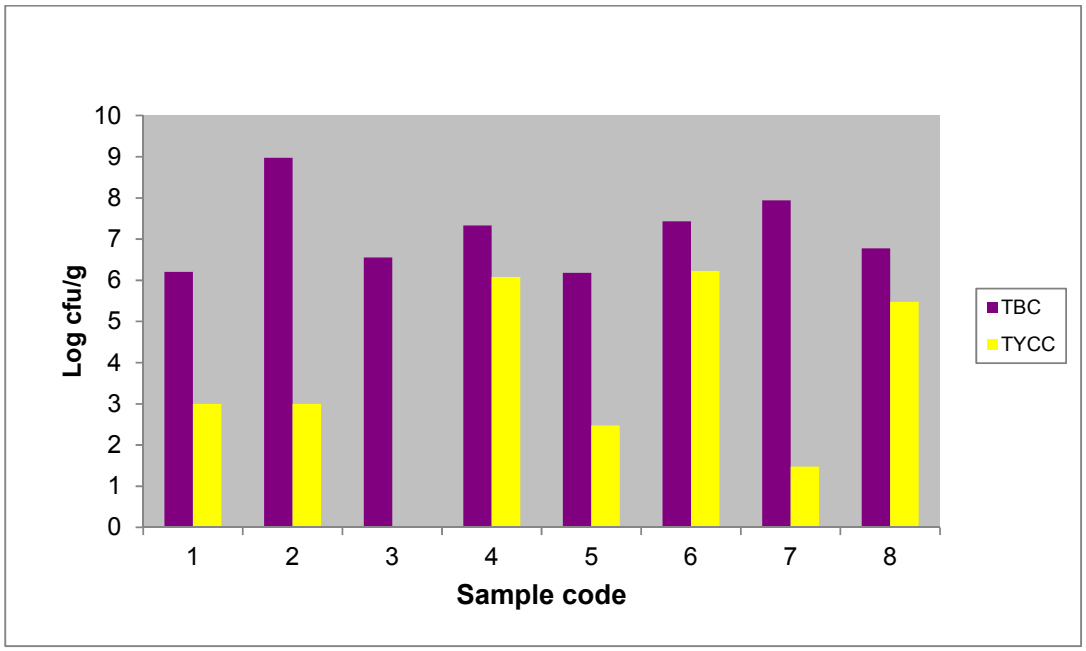


Figure 3.5. Bacterial counts of fresh feather waste samples before burial from plant B incubated at 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h.

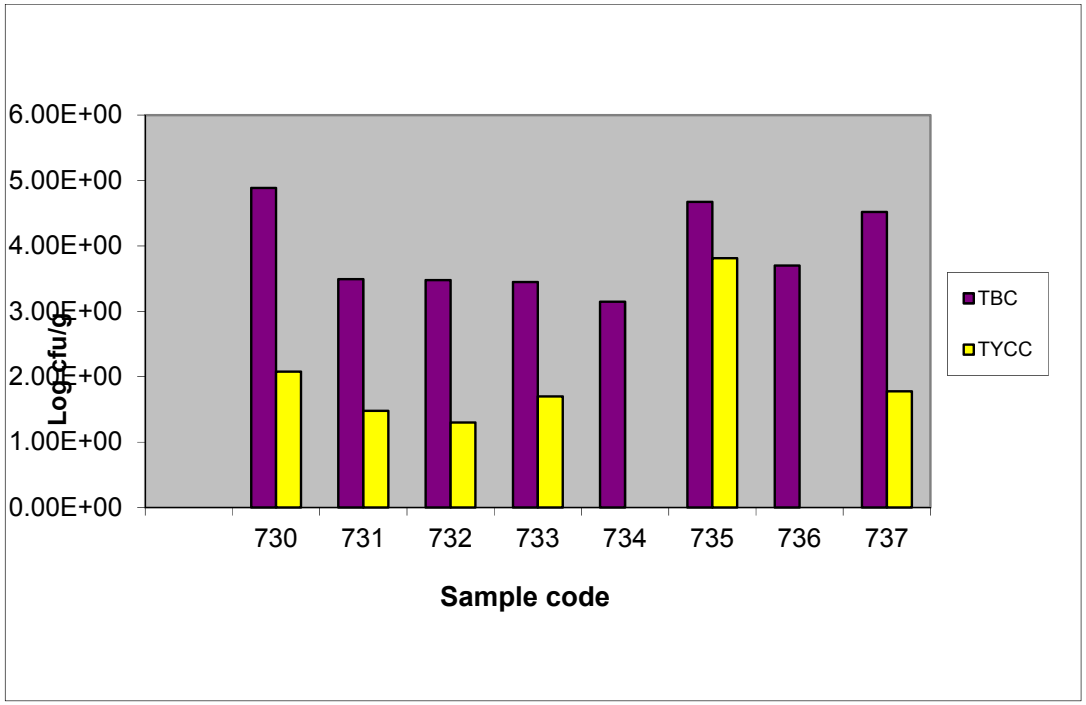


Figure 3.6. Bacterial counts of feather meal samples from plant C incubated at 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h.

The overall TYCC was 10.5 log cfu g^{-1} (45%) out of the overall TBC of 13 log cfu g^{-1}. Four hundred and sixty-six yellow colonies representing the TYCC were picked up, purified by the streaking method and screened for *Chryseobacterium*. Preliminary screening of the isolates showed that 6.1 log cfu g^{-1} of the TYCC were presumptive *Chryseobacterium* isolates. This added up to 37% of the TYCC and was represented by 35 isolates.

3.3.3. *BIOLOG Gen II identification system*

The thirty-five presumptive *Chryseobacterium* isolates, five from poultry processing plant A, 14 from B and 16 from C, were subjected to the BIOLOG Gen II identification system (BIOLOG Inc., Hayward, CA, USA) for further confirmation of the identity of *Chryseobacterium* species. The GN-NENT protocol was followed since the isolates were oxidase positive. The results of the BIOLOG Gen II identification system are shown in Table 3.4. The Gen II microplates were read visually after incubation at 25 ^{o}C for 16 and 24 h. An identification call was made if the similarity index (SIM) was at least 0.50. The SIM was determined by the BIOLOG Microlog software based on the matches between the test organism's characteristic pattern of purple wells, which constitutes a metabolic fingerprint, and the BIOLOG data base.

Probability values (PROB) were calculated taking into account the SIM values as well as the distance values which are calculated from phenotype based dendrograms. If the SIM is less than 0.50, the identification profile is not acceptable and a "No ID" call is made, but the Microlog software may still suggest the nearest genus or species (BIOLOG Inc., 2001). In Table 3.4, 28 identifications fall under this category and they are shown in parenthesis. The number of unidentified isolates was high, possibly because of some of the limitations of the BIOLOG Gen II identification system. Identifications are only done if the test organism is recognized as being similar to members of the species in the database. The Microlog software also reports new and atypical species as "No ID" (BIOLOG Inc., 2001).

Table 3.4. Phenotypic identification using the BIOLOG Gen II identification system.

Isolate	Source	PROB (%)	SIM	ID/No ID call	Identification
1_F178*	Chicken feather waste; processing plant B	97	0.72	ID	[*Chryseobacterium gleum/indologenes*]
29_FM14	Chicken feather meal; processing plant C	98	0.5	ID	[*Elizabethkingia meningoseptica*]
7_F195*	Chicken feather waste; processing plant B	93	0.5	ID	[*Flavobacterium tirrenicum* (*Chryseobacterium)*]
25_F82*	Chicken feather waste; processing plant A	86	0.51	ID	[*Flavobacterium flevense*]
FM67	Chicken feather meal; processing plant C	91	0.72	ID	[*Burkholderia andropogonis*]
FM38	Chicken feather meal; processing plant C	94	0.74	ID	[*Burkholderia glumae*]
F142	Chicken feather waste; processing plant B	100	0.55	ID	[*Burkholderia andropogonis*]
3_F140C*	Chicken feather waste; processing plant B	-	0.23	No ID	[*Chryseobacterium gleum/indologenes*]
F184	Chicken feather waste; processing plant B	-	0.02	No ID	[*Burkholderia andropogonis*]
23_F73*	Chicken feather waste; processing plant A	-	0.43	No ID	[*Chryseobacterium gleum/indologenes*]
24_F49*	Chicken feather waste; processing plant A	-	0.26	No ID	[*Flavobacterium tirrenicum* (*Chryseobacterium)*]
6_F141B*	Chicken feather waste; processing plant B	-	0.27	No ID	[*Flavobacterium tirrenicum* (*Chryseobacterium)*]
26_F94B*	Chicken feather waste; processing plant A	-	0.03	No ID	[*Flavobacterium flevense*]
27_FM7*	Chicken feather meal; processing plant C	-	0.24	No ID	[*Flavobacterium tirrenicum* (*Chryseobacterium)*]
22_F36*	Chicken feather waste; processing plant A	-	0.16	No ID	[*Chryseobacterium gleum/indologenes*]
F92	Chicken feather meal; processing plant C	-	0.46	No ID	[*Burkholderia glumae*]
23_F73	Chicken feather meal; processing plant C	-	0.44	No ID	[*Burkholderia glumae*]
F79	Chicken feather meal; processing plant C	-	0.43	No ID	[*Sphingomonas paucimobilis* A]
F84	Chicken feather meal; processing plant C	-	0.34	No ID	[*Sphingomonas sanguinis*]

F13B	Chicken feather waste; processing plant B	-	0.17	No ID	[*Aeromonas hydrophila-like* DNA group 3]
F10B	Chicken feather waste; processing plant B	-	0.27	No ID	[*Aeromonas schubertii* DNA group 12]
F20	Chicken feather waste; processing plant B	-	0.44	No ID	[*Aeromonas allosaccharophila*]
F166	Chicken feather waste; processing plant B	-	0.35	No ID	[*Aeromonas hydrophila* DNA group 1]
F168	Chicken feather waste; processing plant B	-	0.5	No ID	[*Cardiobacterium hominis*]
2_F143C*	Chicken feather waste; processing plant B	-	0.03	No ID	[*Flavobacterium tirrenicum* (*Chryseobacterium)*]
F41B	Chicken feather waste; processing plant B	-	0.31	No ID	[*Pedobacter heparinus*]
F167	Chicken feather waste; processing plant B	-	0.23	No ID	[*Pasteurella multocida* ss *multocida*]
F56	Chicken feather meal; processing plant C	-	0.37	No ID	[*Rhizobium rhizogenes*]
F57	Chicken feather meal; processing plant C	-	0.44	No ID	[*Rhizobium rhizogenes*]
F41A	Chicken feather meal; processing plant C	-	0.27	No ID	[*Vibrio tubiashii*]
FM12	Chicken feather meal; processing plant C	-	0.17	No ID	[*Vibrio furnissii*]
F69	Chicken feather meal; processing plant C	-	0.11	No ID	[*Vibrio furnissii*]
FM33	Chicken feather meal; processing plant C	-	0.28	No ID	[*Vibrio cincinatiensis*]
28_FM17*	Chicken feather meal; processing plant C	-	0.23	No ID	[*Chryseobacterium gleum/indologenes*]
FM35	Chicken feather meal; processing plant C	-	-	No ID	No ID

*, Presumptive *Chryseobacterium* isolates selected for further investigation during subsequent studies.

Twenty percent (7/35) of the isolates were successfully identified. Four isolates (1_F178, 29_FM14, 7_F195, 25_F82) belonged to the family *Flavobacteriaceae*. The other three were identified as species of the genus *Burkholderia*. A study by Riffel *et al*. (2003) isolated *Chryseobacterium* sp. strain kr6 from poultry feather waste but bacterial counts were not done. Another study by de Beer and Hugo (2010) focusing on potential sources of *Chryseobacterium* sp. contamination during poultry processing reported 17% total yellow colonies counted from raw chicken portions after the brine injection process of which 11.8% was *Chryseobacterium* isolates. Twelve isolates were selected for subsequent taxonomic studies based on their BIOLOG identifications or, if the SIM was less than 0.50, the nearest match compared to the BIOLOG data base.

3.4. Conclusions

Yellow-pigmented isolates were successfully obtained from chicken feather waste and feather meal samples from poultry processing plants using non-selective media. It was possible to further screen for *Chryseobacterium* species using conventional methods and the BIOLOG Gen II microbial identification system.

Standard Plate Count Agar had higher counts for both TBC and TYCC compared to Nutrient Agar at 4 ^{o}C for 10 days and 4 ^{o}C for 48 h then 25 ^{o}C for 48 h. The optimum incubation regime for the yellow colonies was 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h. The yellow-pigmented strains (which include *Chryseobacterium*) did not survive well after being buried one metre below the surface for more than one week. The overall TYCC was 45% of the TBC while presumptive *Chryseobacterium* isolates constituted 37% of the TYCC. A total of twelve isolates were selected for subsequent studies based on analysis of data from the BIOLOG Gen II identification system.

CHAPTER 4

CLASSIFICATION OF *Chryseobacterium* STRAINS ISOLATED FROM RAW CHICKEN AND CHICKEN FEATHER WASTE IN POULTRY PROCESSING PLANTS

ABSTRACT

Ten yellow-pigmented strains isolated from chicken feather waste, two from chicken feather meal and 17 from a previous study (de Beer, 2005), all isolated from poultry processing plants in South Africa, were screened and identified using 16S rRNA sequencing. Fourteen of the 29 isolates were identified as belonging to the genus *Chryseobacterium* by 16S rRNA sequencing. Seven isolates (namely; 5_R23647, 8_R23573, 9_R23581 and 10_R23577 from raw chicken and 1_F178, 6_F141B and 7_F195 from chicken feather waste) that belonged to the genus *Chryseobacterium* and having ≤97% 16S rRNA gene sequence similarity were selected for further 16S rRNA sequencing with two additional primers and subjected to phylogenetic analysis. The isolates were characterized using conventional phenotypic methods. They were also profiled and identified using the BIOLOG Omnilog Gen III identification system. Six out of the seven isolates were identified as belonging to the genus *Chryseobacterium* by the BIOLOG Omnilog Gen III identification system. Four of the nine reference strains used (*C. gleum*, *C. indologenes*, *C. piscium* and *C. scophthalmum*) were correctly identified by the Omnilog system. The others could not be correctly identified because they were not in the Omnilog data base. The seven unknown strains grouped into four possible new species represented by the following strains: 1_F178; 5_R23647; 6_F141B and 7_F195; and 8_R23573, 9_R23581 and 10_R23577. All strains require further polyphasic investigations to have full evidence for description as new species.

4.1. Introduction

The comprehensive characterization of a strain is fundamental in prokaryotic taxonomy and systematics. Taxonomy may be defined as the theory and practice of classifying organisms while systematics is broader, including evolutionary and phylogenetic components (Prakash *et al*., 2007). It provides a reference system for all biological knowledge. Classification is the organization of large numbers of

individual strains into an orderly framework (Gevers *et al*., 2006). Historically, there was strong emphasis on morphological, physiological and biochemical properties for classification (Tindall *et al*., 2010). Advances in methods of studying prokaryotes led to molecular systematics. The 16S rRNA gene sequence analysis is a powerful tool that has provided a basis for studies of the natural relationships among prokaryotes and its application revolutionized their classification (Kämpfer, 2010). However, the classification system based on this gene alone has not solved the taxonomic problems among and within genera. Consequently, phenotypic characterization that is sometimes considered as being "traditional" remains key in determining whether a strain belongs to a known taxon or constitutes a novel one (Kämpfer, 2010).

The genus *Chryseobacterium* was described by Vandamme *et al.* (1994a) and it emerged as the taxonomy of the flavobacteria evolved. This group of flavobacteria was formerly also known as CDC Group IIb and was originally described by King (1959). The original *Flavobacterium* genus was heterogeneous, consisting of a variety of species and even genera. The importance of phenotypes was demonstrated by Holmes in 1992 when he suggested that the flavobacteria should be divided into four natural groups based on phenotypic characteristics and habitat. The first group was later reclassified as the genus *Chryseobacterium* and the other three groups formed the basis of *Flavobacteriaceae* genera such as *Myroides* (group 2; Vancanneyt *et al.,* 1996), *Sphingobacterium* (group 3; Yabuuchi *et al.,* 1983; Takeuchi and Yokota, 1992) and *Bergeyella* (group 4; Vandamme *et al.,* 1994a).

The first aim of this study was to determine whether there were *Chryseobacterium* strains among 29 bacterial isolates in this study. These isolates included ten unidentified isolates from chicken feather waste, two from chicken feather meal (see Chapter 3) and another 17 unidentified *Chryseobacterium*-like isolates obtained from raw chicken after having been subjected to SDS-PAGE analysis in a previous study at the University of the Free State's Food Science Department (de Beer, 2005). Techniques used to characteriize the 29 isolates included 16S rRNA sequencing and identification, conventional phenotypic methods, as well as profiling and identification using the BIOLOG Omnilog Gen III identification system. Briefly stated, the isolates were investigated as follows: Preliminary 16S rRNA gene sequence analysis was done using the forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), and the

reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). Seven isolates whose BLAST results indicated that they belonged to the genus *Chryseobacterium* were selected for sequencing of the almost complete 16S rRNA fragment using two more internal bidirectional primers 341F (5'-CTTATACGGGAGGCAGCAG-3') and 517R (5'-ATTACCGCGGCAGCAG-3').

The second aim was to optimally characterize the seven bacterial strains to more accurately reflect their natural relatedness by using conventional (traditional) tests and the BIOLOG Omnilog gen III identification system. The third aim was to determine if the seven unidentified strains could be regarded as possible representatives of new species based on phylogenetic affiliations and phenotypic traits.

4.2. Materials and methods

4.2.1. *Cultures used and their maintenance*

The isolates and reference strains used in this part of the study are given in Tables 4. 1 and 4. 2 respectively. The chicken feather waste isolates were isolated previously (see Chapter 3) from two poultry processing plants in Bloemfontein (five isolates each from Abattoirs A and B), and one poultry processing plant in Johannesburg (two isolates). The 17 bacterial strains were isolated from raw chicken portions during the study of de Beer (2005) at a poultry processing plant in Bloemfontein (Abattoir A). The reference strains used in the present study were procured from international culture collections (DSMZ and LMG) in a freeze dried state in ampoules. For shorter-term maintenance, the isolates were freeze-dried on 5 mm diameter filter paper discs in sealed Petri dishes and stored at -20 ^{o}C. Before use the strains were reactivated in 10 ml Nutrient Broth (Oxoid CM67). Purity was checked by streaking on Nutrient Agar (Oxoid CM003) and Gram-staining. Incubation was at 25 ^{o}C for 48 h. The pure cultures on Nutrient Agar slants were stored at 4 ^{o}C for short-term maintenance and re-streaked every 4 to 6 weeks.

4.2.2. *16S rRNA gene sequencing*

Table 4. 1. Alpha-numeric code designations of the isolates used, source and year of isolation.

Isolate	Source	Place	Date
1_F178	Chicken feather waste	Bloemfontein (Abattoir B)	2010
2_F143C	Chicken feather waste	Bloemfontein (Abattoir B)	2010
3_F140C	Chicken feather waste	Bloemfontein (Abattoir B)	2010
6_F141B	Chicken feather waste	Bloemfontein (Abattoir B)	2010
7_F195	Chicken feather waste	Bloemfontein (Abattoir B)	2010
22_F36	Chicken feather waste	Bloemfontein (Abattoir A)	2009
23_F73	Chicken feather waste	Bloemfontein (Abattoir A)	2009
24_F49	Chicken feather waste	Bloemfontein (Abattoir A)	2009
25_F82	Chicken feather waste	Bloemfontein (Abattoir A)	2009
26_F94B	Chicken feather waste	Bloemfontein (Abattoir A)	2009
27_FM7	Chicken feather meal	Johannesburg abattoir	2009
28_FM17	Chicken feather meal	Johannesburg abattoir	2009
5_R23647	Chicken portion	Bloemfontein (Abattoir A)	2002/3
8_R23573	Chicken portion	Bloemfontein (Abattoir A)	2002/3
9_R23581	Chicken portion	Bloemfontein (Abattoir A)	2002/3
10_R23577	Chicken portion	Bloemfontein (Abattoir A)	2002/3
11_R23605	Chicken portion	Bloemfontein (Abattoir A)	2002/3
12_R23547	Chicken portion	Bloemfontein (Abattoir A)	2002/3
13_R23603	Chicken portion	Bloemfontein (Abattoir A)	2002/3
14_R23604	Chicken portion	Bloemfontein (Abattoir A)	2002/3
15-R23627	Chicken portion	Bloemfontein (Abattoir A)	2002/3
16_R23500	Chicken portion	Bloemfontein (Abattoir A)	2002/3
17_R23590	Chicken portion	Bloemfontein (Abattoir A)	2002/3
18_R23628	Chicken portion	Bloemfontein (Abattoir A)	2002/3
19_R23599	Chicken portion	Bloemfontein (Abattoir A)	2002/3
21_R23585	Chicken portion	Bloemfontein (Abattoir A)	2002/3
30_R23597	Chicken portion	Bloemfontein (Abattoir A)	2002/3
34_R23602	Chicken portion	Bloemfontein (Abattoir A)	2002/3
36_R23578	Chicken portion	Bloemfontein (Abattoir A)	2002/3

Table 4.2. Reference strains (nearest phylogenetic neighbours) used for conventional phenotypic tests.

Genus and species	Culture collection[a]	Source of isolation	Reference
Chryseobacterium shigense	DSM 17126^{T}	Lactic acid beverage	Shimomura *et al*., 2005
C. luteum	LMG23785^{T}	Phyllosphere of grasses	Behrendt *et al*., 2007

[a], DSM, Deutsche Sammlung von Mikro-organismen; LMG, Laboratorium voor Microbiologie, Ghent, Belgium.

All 29 isolates were subjected to whole-cell polymerase chain reaction (PCR) amplification of the 16S rRNA gene. Briefly stated, a 50 µl reaction volume was set up. The reaction mixture contained a few cells that were suspended in 25 µl of double distilled water and heated at 96 ^{o}C for 10 min. in an Eppendorf Mastercycler Temperature Gradient Personal Thermal Cycler to rupture cells and expose rRNA. A sufficient quantity of cells was transluscent in the 25 µl of double distilled water. The other 25 µl reaction mixture was constituted to make a 0.1 X PCR mixture in the final 50 µl. The final reaction volume contained PCR buffer (0.1 X), $MgCl_2$ (1.5 mM), dNTPs (0.02 mM), 100 pM each of the forward primer, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer, 1492R (5'-GGTTACCTTGTTACGACTT-3') corresponding to the *E. coli* 16S rRNA numbering system, taq polymerase and double distilled water to top up to volume. Thermal cycling was carried out in an Eppendorf Mastercycler Temperature Gradient Personal Thermal Cycler programmed as follows: initial denaturation at 94 ^{o}C for 2 min., 35 cycles of denaturing at 94 ^{o}C for 30 sec., annealing at 53 ^{o}C for 30 sec. and elongation at 72 ^{o}C for 1.5 min. Final elongation was done at 72 ^{o}C for 7 min. The PCR products were visualized on a 1% agarose gel to which Gold View stain was added. The agarose gel was prepared using TAE buffer comprising of 100 mM Tris (2-amino-2-hydroxymethyl-1,3 propanediol) HCl (pH 8.0); 50 mM EDTA (disodium ethylenediaminetetraacetic acid) and 100 mM glacial acetic acid. Gel loading volumes consisted of 5 of the PCR products to which 1.5 of 6 X loading buffer were added. Electrophoresis was done for 30 min. at 9 mV cm^{-1}. The DNA marker used was Fermentas O'gene Ruler™.

The approximately 1500 bp amplicons were purified using a Biospin Gel Extraction Kit (Bioflux) according to manufacturer's instructions. Sequencing was done in two stages with an ABI BigDye® Terminator v1.1 sequencing cycler (Applied Biosystems®) using the ddNTP chain termination method according to manufacturer's instructions. Firstly, the external primers 27F and 1492R were used to obtain sequences that were used in the preliminary identification of the isolates. The sequences were manually edited and analysed using Gene Pro 4.8 (Drummond *et al*., 2009) and compared with sequences on Genbank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST). Secondly, seven highly proteolytic isolates (data not shown) whose BLAST results

indicated that they belonged to the genus *Chryseobacterium* were selected for further sequencing to obtain the almost complete 16S rRNA fragment. For this sequencing step, additional internal primers 341F (5'-CTTATACGGGAGGCAGCAG-3') and 517R (5'-ATTACCGCGGCAGCAG-3') were used. The sequences were manually edited and analysed as previously described. Contigs were assembled using four sequences for each isolate.

Comparative gene sequence homologies of the final sequences were analysed using CLUSTALW2 multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequences were then compared with sequences retrieved from Genbank as described previously and aligned using the CLUSTAL_X version 2.0 (Larkin *et al*., 2007). Phylogenetic and molecular evolutionary analyses were conducted with MEGA software version 4 (Tamura *et al*., 2007) using the neighbour joining method with the Kimura two (KP2) parameter distance measure to determine the relationship of the isolates under study to the type strains of the 58 *Chryseobacterium* species known to date. Confidence values were estimated from bootstrap analysis of 1 000 replicates.

4.2.3. *Conventional phenotypic tests*

4.2.3.1. *Strains used*

The 29 strains used and their designations are provided in Table 4.3. They were isolated from samples obtained from two chicken processing plants in Bloemfontein, Free State Province (designated A and B) and one processing plant from Johannesburg, Gauteng Province, South Africa. Table 4.4 shows the two reference strains used in this part of the study. They were selected on the basis of being the nearest phylogenetic neighbours to the isolates.

4.2.3.2. *Characterization*

A range of phenotypic tests that were selected on the basis of being able to differentiate between members of the genus *Chryseobacterium* was used. The tests used were according to the minimal standards for the description of new genera and

Table 4.3. The sources and year of isolation of bacterial strains for phenotypic characterization obtained from chicken feather waste and raw chicken portions.

Isolate	Source	Place	Date
1_F178	Chicken feather waste	Bloemfontein (Abattoir B)	2010
6_F141B	Chicken feather waste	Bloemfontein (Abattoir B)	2009
7_F195	Chicken feather waste	Bloemfontein (Abattoir B)	2010
5_R23647	Chicken portion	Bloemfontein (Abattoir A)	2002/3
8_R23573	Chicken portion	Bloemfontein (Abattoir A)	2002/3
9_R23581	Chicken portion	Bloemfontein (Abattoir A)	2002/3
10_R23577	Chicken portion	Bloemfontein (Abattoir A)	2002/3

Table 4.4. *Chryseobacterium* reference strains used for phenotypic characterization.

Genus and species	Culture collection[a]	Source of isolation	Reference
C. shigense	DSM 17126^T	Lactic acid beverage	Shimomura *et al*., 2005
C. luteum	LMG23785^T	Phyllosphere of grasses	Behrendt *et al*., 2007
C. ureilyticum	DSM18017^T	Beer-bottling plant	Herzog *et al*., 2008
C. oranimense	DSM19055^T	Raw cow's milk	Hantsis-Zacharov *et al*., 2008b
C. gleum	NCTC 11432^T	Human vaginal swab	Holmes *et al.,* 1984b
C. indologenes	LMG8337^T	Human trachea at autopsy	Yabuuchi *et al.,* 1983
C. piscium	LMG23089^T	Fresh marine fish	de Beer *et al*., 2006
C. balustinum	NCTC 11212 ^{T}	Heart blood of fresh water fish (dace, *Leuciscus leuciscus*)	Holmes *et al.,* 1984a
C. scophthalmum	LMG 13028^T	Gills of diseased turbot (*Scophthalmus maximus*)	Mudarris *et al.,* 1994

[a], DSM, Deutsche Sammlung von Mikro-organismen; LMG, Laboratorium voor Microbiologie, Ghent, Belgium; NCTC, National Collection of Type Cultures.

cultivable species of the family *Flavobacteriaceae* (Bernardet *et al*., 2002). The isolates were incubated at 25 ^{o}C and the biochemical tests were performed according to Cowan (1974) and MacFaddin (1980) unless otherwise stipulated under details for each method.

Colonial morphology was observed on Nutrient Agar. Strains were streaked on Nutrient Agar and incubated for 48 h. Gram-staining and cell morphology were done according to MacFaddin (1980). The presence or absence of fruity odour was noted. The non-staining (KOH) Gram-staining reaction was done according to Buck (1982). The production of oxidase, catalase and phosphatase enzymes was determined according to MacFaddin (1980).

The production of flexirubin-type pigments was carried out according to Bernardet *et al*. (2002). A small mass of bacterial cells was smeared on a glass slide on a white background and flooded with 20% (w/v) potassium hydroxide. Colonies having a flexirubin type pigment exhibited an immediate colour shift from yellow to red, purple or brown and reverted to their initial colour when flooded by an acidic solution (1 N HCl) once the excess of KOH was removed (Reichenbach, 1989). Motility was determined by phase contrast examination of wet mounts from Nutrient Broth (Oxoid, CM67). Gliding motility was determined according to Jooste *et al*. (1985). Fluorescence was demonstrated on King's medium B (Cowan, 1974).

Biochemical characteristics were determined using standardized growth suspensions from 24 h Nutrient Broth cultures. Cells were harvested by centrifugation at 10 000 rpm for 10 min using a Beckmann J2-21 centrifuge and the pellets were washed twice before being re-suspended using 0.1 M phosphate buffer pH 7.0. The suspensions were standardized in comparison with a McFarland number 2 density standard (Difco 0691326) using 0.1 M phosphate buffer pH 7.0. Inoculations were conducted using a multi-inoculation device (Jooste, 1985).

The following range of phenotypic tests were carried out according to Cowan (1974) and MacFaddin (1980): oxidative or fermentative metabolism of glucose; methyl red and Voges Proskauer reactions; gluconate oxidation; potassium cyanide tolerance;

malonate utilization; growth in 0 to 5% (w/v) sodium chloride; growth at 5, 25, 30, 35, 37 and 42 ^{o}C; growth on Cetrimide agar (Merck 5284), MacConkey agar (Oxoid CM0007), Simmon's Citrate Agar (Oxoid CM155); reduction of 0.4% selenite (Holmes *et al*., 1975); nitrate and nitrite reduction; Production of acid from 10% (w/v) glucose and lactose; alkaline reaction on Christensen's Citrate Agar (Holmes *et al*., 1975); production of ammonia from arginine; lysine decarboxylase, ornithine decarboxylase, deoxyribonuclease (Oxoid CM321+0.01% toluidine blue), β-galactosidase (ONPG), hydrogen sulphide (TSI method), indole (Kovac's reagent), 3-ketolactose, phenylalanine deaminase, urease on Christensen's Urea Agar (Richard and Kiredjian, 1995; Hugo, 1997); hydrolysis of esculin (Yabuuchi *et al*., 1990), casein, gelatine (plate method), starch (West and Colwell, 1984), Tween 20, Tween 80 (West and Colwell, 1984), tyrosine (Barrow and Feltham, 1993); acid production in adonitol, L-arabinose, dulcitol, ethanol and D-xylose. The sugars were used at a final concentration of 1% (w/v).

4.2.4. *BIOLOG Omnilog Gen III system*

In addition to the conventional tests, all strains were profiled using the BIOLOG Omnilog Gen III identification system (BIOLOG Inc., Hayward, CA, USA) according to the manufacturer's instructions. The microtitre plates were incubated at 25 ^{o}C for 22 h. The plates were read using a BIOLOG microstation reader with wavelength set at 595 nm.

4.3. Results and discussion

4.3.1. *PCR amplicons*

Figures 4.1a, b, c, and d, show the electrophoregrams of the PCR amplicons for the 29 isolates' 16S rRNA region. All the isolates gave the expected ~1500 bp band after optimization of the PCR cycle by increasing the annealing temperature from 49 ^{o}C to 53 ^{o}C. All the isolates that yielded low PCR products as shown by the agarose gel electrophoresis were re-assayed using fresh cultures. The PCR products were stored at -20 ^{o}C until they were sequenced within seven days.

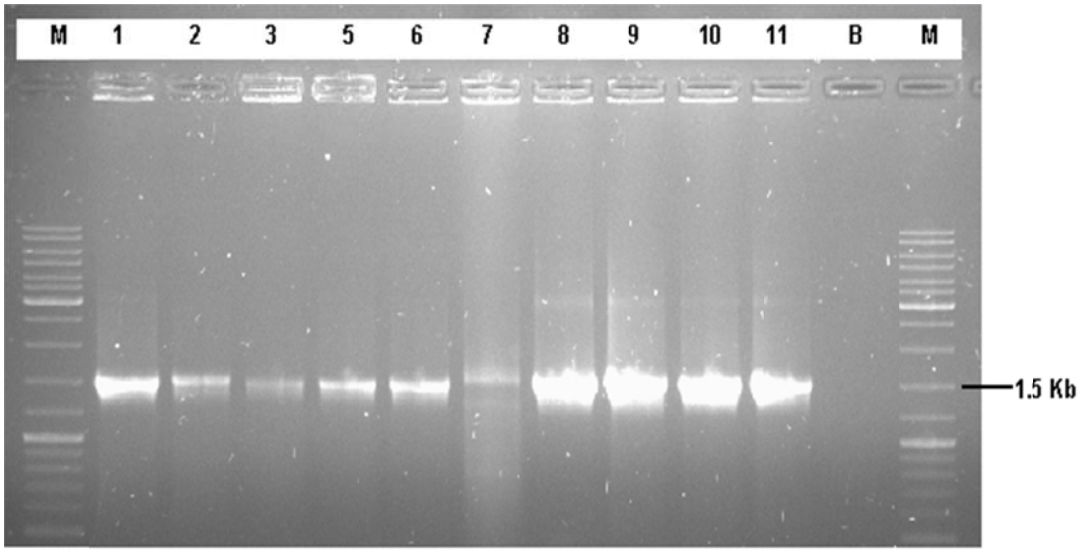


Figure 4.1a. Electrophoregram of the ~1500 bp PCR products for the isolates' 16S rRNA region. Isolates 1, 1_F178; 2, 2_F143C; 3, 3_F140C; 5, 5_R23647; 6, 6_F141B; 7, 7_F195; 8, 8_R23573; 9, 9_R23581; 10, 10_R23577; 11, 11_R23605; B, Negative control; M, DNA molecular marker.

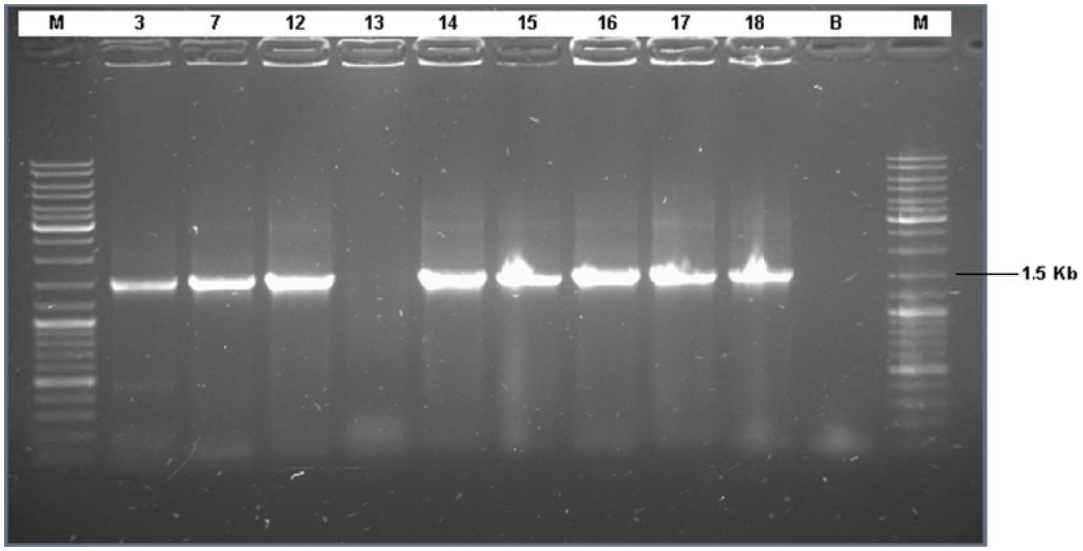


Figure 4.1b. Electrophoregram of the ~1500 bp PCR products for the isolates' 16S rRNA region. Isolates 3, 3_F140C; 7, 7_F195; 12, 12_R23547; 13, 13_R23603; 14, 14_R23604; 15, 15_R23627; 16, 16_R23500; 17, 17_R23590; 18, 18_R23628; B, Negative control; M, DNA molecular marker.

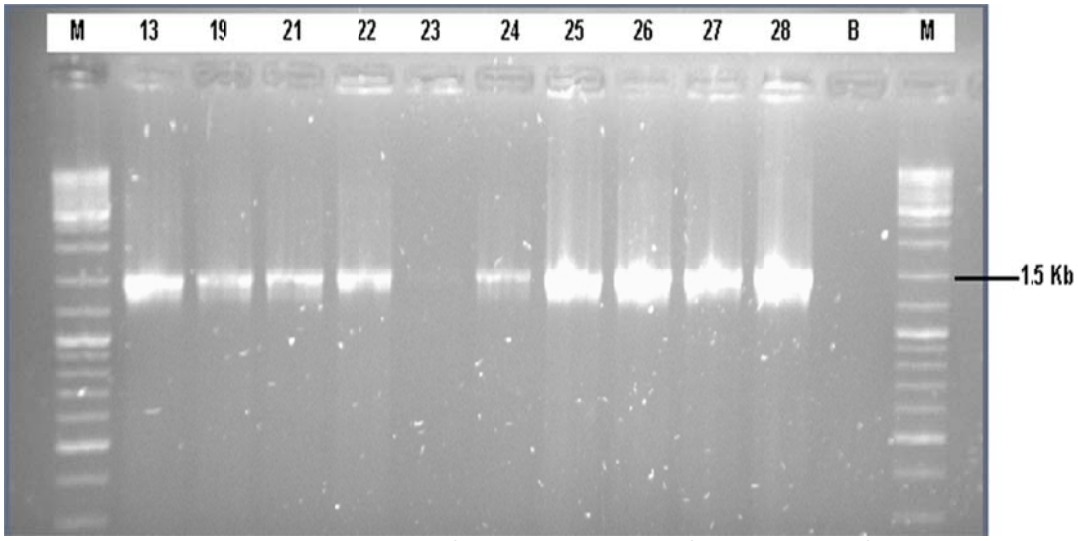


Figure 4.1c. Electrophoregram of the ~1500 bp PCR products for the isolates' 16S rRNA region. Isolates 13, 13_R23603; 19, 19_R23599; 21, 21_R23585; 22, 22_F36; 23, 23_F73; 24, 24_F49; 25, 25_F49B; 26, 26_F94B; 27, 27_FM7; 28, 28_FM17; B, Negative control; M, DNA molecular marker.

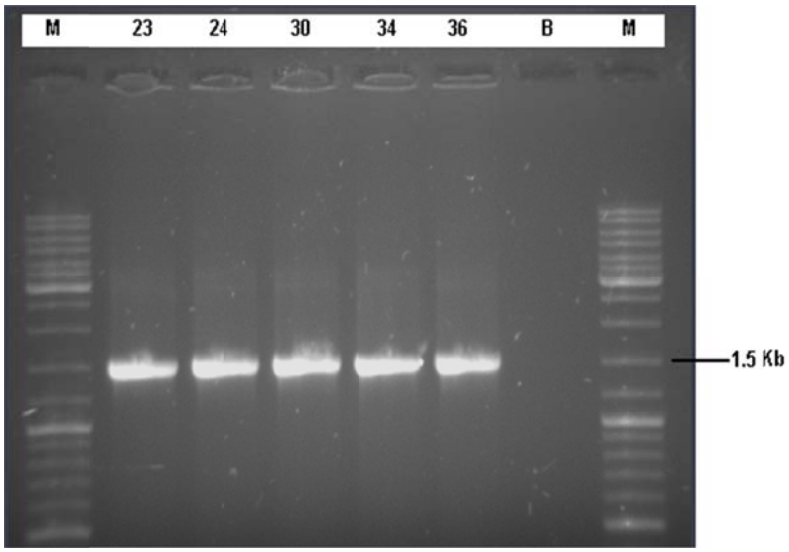


Figure 4.1d. Electrophoregram of the 1500 bp PCR products for the isolates' 16S rRNA region. Isolates 23, 23_F73; 24, 24_F49; 30, 30_R23597; 34, 34_R23602; 36, 36_R23578; B, Negative control; M, DNA molecular marker.

4.3.2. *Preliminary 16S rRNA sequencing identifications*

Table 4.5 shows the summary of the 16S rRNA identifications of the 29 isolates investigated. Of the 29 isolates initially sequenced in this part of the study, 6 isolates (22_F36, 23_F73, 11_R23605, 14_R23604, 15_R23627 and 30_R23597) could not be sequenced successfully. Poor quality sequences were obtained after three attempts and a decision was taken not to further investigate these isolates. It was later concluded that the poor quality sequences may have been due to a low template concentration (less than 10-40 ng) as revealed by nanodrop measurements (data not shown). Six isolates (3_F140C, 25_F82, 28_FM17, 21_R23585, 34_R23602 and 36_R23578) belonged to the genus *Pseudomonas*. Three isolates (2_F104C, 26_F94B and 27_FM7) were identified as *Sphingomonas paucimobilis*, while 14 isolates (1_F178, 6_F141B, 7_F195, 24_F49, 5_R23647, 8_R23573, 9_R23581, 10_R23577, 12_R23547, 13_R23603, 16_R23500, 17_R23590, 18_R23628 and 19_R23599), belonged to the genus *Chryseobacterium*.

The isolates 1_F178, 6_F141B and 7_F195 were isolated from chicken feather waste and isolates 5_R23647, 8_R23573, 9_R23581 and 10_R23577 were isolated from raw chicken portions in a chicken processing plant in Bloemfontein.

A summary of the multiple sequence analysis of the isolates using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) is shown in Table 4.6. Comparative sequence analysis revealed 99.0% 16S rRNA gene sequence similarity among the strains 8_R23573, 9_R23581 and 10_R23577. This demonstrated their high phylogenetic relatedness. The 16S rRNA gene sequence similarity between these three strains and the others (1_F178, 5_R23647, 6_F141B and 7_F195) ranged from 47 to 97% demonstrating that these strains had low phylogenetic relatedness when the generally accepted threshold of 97% is considered (Wayne *et al.,* 1987). Isolate 1_F178 had 97% 16S rRNA gene sequence similarity compared to isolate 6_F141B. This was a borderline value since all similarity values above 97% are generally indicative of same species status and DNA-DNA hybridization is required to confirm separate species status. The rest of the sequence similarity permutations among the isolates ranged from 47 to 96% showing their low relatedness.

Table 4.5. Summary of the 16S rRNA identifications of the 29 isolates investigated.

Isolate	Identification	Similarity (%)
1_F178	*Chryseobacterium* jll sp	94
2_F143C	*Sphingomonas paucimobilis*	100
3_F140C	*Pseudomonas* sp.	100
6_F141B	*Chryseobacterium* sp.	95
7_F195	*Chryseobacterium* sp.	98
22_F36	No ID	-
23_F73	No ID	-
24_F49	*Chryseobacterium vrystaatense* strain R23533	97
25_F82	*Pseudomonas* sp.	98
26_F94B	*Sphingomonas paucimobilis*	99
27_FM7	*Sphingomonas paucimobilis*	99
28_FM17	*Pseudomonas* sp.	91
5_R23647	*Chryseobacterium* sp.	97
8_R23573	*Chryseobacterium* sp.	96
9_R23581	*Chryseobacterium* sp.	96
10_R23577	*Chryseobacterium* sp.	96
11_R23605	No ID	-
12_R23547	*Chryseobacterium* sp.	99
13_R23603	*Chryseobacterium vrystaatense* R23533	99
14_R23604	No ID	-
15-R23627	No ID	-
16_R23500	*Chryseobacterium vrystaatense* R23533	98
17_R23590	*Chryseobacterium vrystaatense* R23533	98
18_R23628	*Chryseobacterium vrystaatense* R23533	99
19_R23599	*Chryseobacterium vrystaatense* R23533	98
21_R23585	*Pseudomonas* sp.	99
30_R23597	No ID	-
34_R23602	*Pseudomonas* sp.	96
36_R23578	*Pseudomonas* sp.	97

Table 4.6. Multiple sequence analysis of strains 1_F178, 5_R23647, 6_F141B, 7_F195, 8_R23573, 9_R23581 and 10_R23577 using CLUSTALW2 (http://www.ebi.ac.uk/ Tools/msa/clustalw2).

Sequence A Isolate	Sequence B Isolate	Similarity (%)
10_R23577	9_R23581	99.0
10_R23577	8_R23573	99.0
9_R23581	8_R23573	99.0
1_F178	6_F141B	97.0
10_R23577	7_F195	96.0
1_F178	5_R23647	96.0
7_F195	9_R23581	96.0
7_F195	8_R23573	96.0
5_R23647	6_F141B	94.0
1_F178	7_F195	50.0
10_R23577	6_F141B	49.0
5_R23647	7_F195	49.0
5_R23647	9_R23581	49.0
9_R23581	6_F141B	49.0
8_R23573	6_F141B	49.0
10_R23577	1_F178	48.0
10_R23577	5_R23647	48.0
1_F178	9_R23581	48.0
5_R23647	8_R23573	48.0
7_F195	6_F141B	48.0
1_F178	8_R23573	47.0

The seven isolates were selected for sequencing with an additional two internal bidirectional primers to obtain the complete approximately 1500 bp 16S rRNA segment. The four sequences obtained from each isolate were combined to make the final consensus contig for that isolate.

4.3.3. *16S rRNA gene sequence analysis*

The BLAST search results for the edited consensus sequences compared to the sequences of recognised *Chryseobacterium* type species on the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) are shown in Table 4.7. The maximum percent identity (Max. % identity) corresponds to a match to the subject sequence with the highest percentage of identical bases. Hence it represents the percent similarity (% similarity) which is required to assign an isolate to a certain species or genus. The query coverage represents the fraction of the query sequence that matches a subject sequence. The query coverage for all the isolates ranged from 96 to 100%. The E-value is the expected value. It is used as a convenient way to create a significant threshold when reporting % similarity. The closer to zero the E-value, the more significant it is. The E-value obtained for all the isolates was 0.00 indicating that all the % identities were significant.

The bacterial isolates from raw chicken namely 8_R23573, 9_R23581 and 10_R23577 had the highest similarities to *C. shigense* DSM 17126^T and C. *luteum* DSM18605^T was the second most closely related species to these isolates. The % similarity of isolate 8_R23573 to *C. shigense* DSM 17126^T was 99.22 with a query coverage of 96%. Isolate 8_R23573 showed a similarity of 98.31% to C. *luteum* DSM18605^T with a query coverage of 98%.

Table 4.7. The BLAST search results for the seven isolates' sequences compared to the sequences of recognised *Chryseobacterium* type species on the NCBI GenBank database (http://www.ncbi.nlm.nih.gov).

Isolate	Accession	Description	Query coverage (%)	E. value	Max % Identity
1_F178	NR_044300.1	*Chryseobacterium jejuense* strain JS17-8^{T} 16S ribosomal RNA, partial sequence	96	0.0	98.99 (1371/1385)
	AM232806.1	*Chryseobacterium ureilyticum* partial 16S rRNA gene, type strain F-Fue-04IIIaaaa^{T}	99	0.0	97.90 (1400/1430)
	NR_044334.1	*Chryseobacterium aquifrigidense* strain CW9^{T} 16S ribosomal RNA, partial sequence	98	0.0	97.66 (1378/1411)
5_R23647	AM040439.1	*Chryseobacterium piscium* partial 16S rRNA gene, strain LMG 23089^{T}	98	0.0	98.85 (1373/1389)
	NR_042926.1	*Chryseobacterium indoltheticum* strain LMG 4025^{T} 16S ribosomal RNA gene, partial sequence	99	0.0	98.71 (1379/1397)
	NR_043253.1	*Chryseobacterium soldanellicola* strain PSD1-4 16S^{T} ribosomal RNA gene, partial sequence	98	0.0	97.69 (1355/1387)
	NR_025386.1	*Chryseobacterium scophthalmum* 16S rRNA gene, strain LMG 13028^{T}	98	0.0	97.61 (1350/1383)
6_141B	NR_042507.1	*Chryseobacterium indologenes* partial 16S rRNA gene, strain LMG 8337^{T}	97	0.0	98.12 (1360/1386)
	NR_042506.1	*Chryseobacterium gleum* strain CCUG 14555 16S ribosomal RNA, partial sequence	98	0.0	97.50 (1367/1402)
	AM232806.1	*Chryseobacterium ureilyticum* partial 16S rRNA gene, strain F-Fue-04IIIaaaa^{T}	100	0.0	97.46 (1382/1418)

	NR_044168.1	*Chryseobacterium oranimense* strain H8 16S^{T} ribosomal RNA, partial sequence	99	0.0	97.18 (1377/1417)
	NR_044334.1	*Chryseobacterium aquifrigidense* strain CW9 16S^{T} ribosomal RNA gene, partial sequence	99	0.0	97.10 (1375/1416)
7_F195	NR_042507.1	*Chryseobacterium indologenes* partial 16S rRNA gene, strain LMG 8337^{T}	96	0.0	98.04 (1354/1381)
	AM232806.1	*Chryseobacterium ureilyticum* partial 16S rRNA gene, type strain F-Fue-04IIIaaaa^{T}	99	0.0	97.40 (1387/1424)
	NR_044168.1	*Chryseobacterium oranimense* strain H8 16S^{T} ribosomal RNA gene, partial sequence	99	0.0	97.05 (1382/1424)
	AM261868.1	*Chryseobacterium hominis* partial 16S rRNA gene, strain NF802^{T}	99	0.0	96.57 (1378/1427)
8_R23573	HQ630670.1	*Chryseobacterium shigense* strain DSM 17126^{T} 16S ribosomal RNA gene	97	0.0	99.22 (1394/1405)
	AM489609.1	*Chryseobacterium luteum* partial 16S rRNA gene, type strain DSM 18605^{T}	98	0.0	98.31 (1397/1421)
	NR_044168.1	*Chryseobacterium oranimense* strain H8 16S^{T} ribosomal RNA gene, partial sequence	99	0.0	97.70 (1404/1437)
	AM232806.1	*Chryseobacterium ureilyticum* partial 16S rRNA gene, type strain F-Fue-04IIIaaaa^{T}	99	0.0	97.57 (1404/1439)
	AJ871397.1	*Chryseobacterium vrystaatense* 16S rRNA gene, type strain R-23566^{T}	98	0.0	97.89 (1391/1421)
9_R23581	HQ630670.1	*Chryseobacterium shigense* strain DSM 17126^{T} 16S ribosomal RNA gene, partial sequence	98	0.0	99.78 (1387/1390)
	AM489609.1	*Chryseobacterium luteum* partial 16S rRNA gene, type strain DSM 18605^{T}	99	0.0	98.72 (1391/1409)

	NR_044168.1	*Chryseobacterium oranimense* strain H8^{T} 16S ribosomal RNA gene, partial sequence	100	0.0	98.37 (1391/1414)
	NR_042370.1	*Chryseobacterium vrystaatense* 16S rRNA gene, type strain R-23566^{T}	99	0.0	98.37 (1386/1409)
	AM232806.1	*Chryseobacterium ureilyticum* partial 16S rRNA gene, type strain F-Fue-04IIIaaaa^{T}	100	0.0	98.09 (1389/1416)
10_R23577	HQ630670.1	*Chryseobacterium shigense* strain DSM 17126^{T} 16S ribosomal RNA gene, partial sequence	97	0.0	99.71 (1387/1391)
	AM489609.1	*Chryseobacterium luteum* partial 16S rRNA gene, type strain DSM 18605^{T} = LMG 23785	98	0.0	98.65 (1389/1408)
	NR_044168.1	*Chryseobacterium oranimense* type strain H8 = DSM 19055 = LMG 24030; 16S ribosomal RNA, partial sequence	99	0.0	98.24 (1397/1422)
	AM232806.1	*Chryseobacterium ureilyticum* partial 16S rRNA gene, type strain F-Fue-04IIIaaaa^{T}	99	0.0	98.04 (1397/1425)
	AJ871397.1	*Chryseobacterium vrystaatense* 16S rRNA gene, type strain R-23566^{T}	98	0.0	98.30 (1384/1408)

The other *Chryseobacterium* type strains that were similar to isolate 8_R23573 were *C. oranimense* H8^T (97.70% similarity at 99% query coverage) and *C. ureilyticum* F-Fue-04IIIaaaa^T (97.57% similarity at 99% query coverage). Isolate 9_R23581 had a 99.78% similarity to *C. shigense* DSM 17126^T with a query coverage of 98%. It had a similarity of 98.65% with *C. luteum* DSM18605^T. The other *Chryseobacterium* type strains that shared ≥97.00% similarity to isolate 9_R23581 were *C. oranimense* strain H8^T, *C. vrystaatense* LMG 22846^T and *C. ureilyticum* F-Fue-04IIIaaaa^T (Table 4.7). Isolate 10_R23577 had a similarity of 99.71% to *C. shigense* DSM 17126^T with a query coverage of 97%. It shared 98.65% similarity with C. *luteum* DSM18605^T. It was also similar to two other type strains, *C. oranimense* strain H8^T and *C. ureilyticum* F-Fue-04IIIaaaa^T at 98.24% and 98.04% similarity respectively (Table 4.7).

The bacterial isolate from raw chicken namely 5_R23647, was most closely related to *C. piscium* LMG23089^T at 98.12% similarity and a query coverage of 97%. The second most closely related species to isolate 5_R23647 was *C. indoltheticum* strain LMG 4025^T with a sequence similarity of 98.71% at 99% query coverage. Isolate 5_R23647 was also closely related to *C. soldanellicola* strain PSD1-4^T and *C. scophthalmum* strain LMG 13028^T at 97.69 and 97.61% respectively.

The 16S rRNA gene sequence similarities of the seven isolates to currently recognised *Chryseobacterium* type species ranged from 97.05 to 99.78%. This exceeded the generally accepted threshold value of 97.0% for the delineation of a novel species. According to Wayne *et al*. (1987) and Stackebrandt and Goebel (1994), values above 97% 16S rRNA gene sequence similarity require DNA-DNA hybridization (DDH) studies to confirm separate species status. Therefore all the seven isolates required DDH studies.

On the other hand, Stackebrandt and Ebers (2006) re-evaluated the correlation between 16S rRNA gene sequence similarity and DNA re-association values and proposed a threshold value of 98.7%. Taking this cut-off value into consideration, isolates 6_F141B and 7_F195 represent a new species while isolates 1_F178, 5_R23647, 8_R23573, 9_R23581 and 10_R23577 still require DDH for confirmation

as strains of novel species. Drancourt *et al*. (2000) proposed a threshold value of 99.0% 16S rRNA gene sequence similarity for novel species delineation. Taking this cut-off value into consideration, isolates 1_F178, 5_R23647, 6_F141B and 7_F195 should be regarded as affiliates of novel species.

However, the discussion of the correlation between 16S rRNA gene sequence similarity and DNA re-association values has been controversial. Novel species with more than 97.0% 16S rRNA gene sequence similarities with their closest phylogenetic neighbours have been reported. *Chryseobacterium vrystaatense* LMG22848^T showed 96.9, 97.1 and 97.1% similarity to *C. joostei*, *C. indologenes* and *C. gleum,* respectively (de Beer *et al*., 2005). *Chryseobacterium shigense* and *C. vrystaatense* were phylogenetic neighbours of *C. luteum* P 456/04^T showing sequence similarities of 98.3 and 97.3% respectively (Behrendt *et al*., 2007). Other high value 16S rRNA gene sequence similarities were reported for the following pairs of type strains: *C. daecheongense* and *C. defluvii* (97.9%; Kim *et al*., 2005b); *C. ureilyticum* F-Fue-04IIIaaaa^T and *C. joostei* LMG 18212^T (98.3%; Herzog *et al*., 2008) and *C. aquifrigidense* compared to *C. gleum* and *C. indologenes* (98.4 and 97.8% respectively; Park *et al*., 2008).

Kämpfer *et al*. (2010a) reported a very high similarity value above 99.0% between *C. arthrosphaerae* and *C. gleum* CCUG 14555^T (99.2%). However, DDH values for all these strains were far below the 70% cut-off value for novel species delineation (Wayne *et al*., 1987).

The lack of universal consensus on the % similarity reflecting the true correlation with DDH data and hence novel genus or species status had the result that the Subcommittee on the Taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria of the International Committee on Systematics of Prokaryotes endorsed DDH data over and above 16S rRNA as mandatory for the description of new species (Bernardet *et al*., 2002; Bernardet *et al.*, 2011).

Figure 4.2 shows the neighbour joining phylogenetic analysis of strains 1_F178, 5_R23647, 6_F141B, 7_F195, 8_R23573, 9_R23581 and 10_R23577 compared to the type strains of currently recognised *Chryseobacterium* species. Phylogenetic

trees were also constructed using two other methods, the maximum parsimony algorithm and UPGMA analysis in the MEGA version 4 software package (Tamura *et al*., 2007; Appendices I and II respectively). It revealed a clear affiliation of all seven isolates to the genus *Chryseobacterium*. Strains 8_R23573, 9_R23581 and 10_R23577 isolated from raw chicken clustered tightly together (bootstrap re-sampling support value of 85%) suggesting that they could belong to the same species. These strains clustered with *C. shigense* GUM Kaj^T as their nearest phylogenetic neighbour.

The stability of this phylogeny was supported by a high bootstrap re-sampling value of 99%. This phylogeny was also obtained using two other treeing methods and was supported by bootstrap re-sampling values above 70% (72% in the maximum parsimony tree and 99% in the UPMGA tree; Appendices I and II respectively). *Chryseobacterium oranimense* and *C. luteum* formed a sub-cluster with strains 8_R23573, 9_R23581, 10_R23577 and *C. shigense* GUM Kaj^T. This phylogeny was also obtained using the other two tree-making methods. However, it was weakly supported since the bootstrap re-sampling values were lower than 70%. *Chryseobacterium shigense* was isolated from a fresh lactic acid beverage in the Shiga Prefecture, Japan (Shimomura *et al*., 2005) and *C. luteum* was isolated from the phylosphere of grasses (Behrendt *et al*., 2007) while *C. oranimense* was isolated from raw milk (Hantsis-Zacharov *et al*., 2008). This reflects the wide diversity of ecological niches associated with members of the genus *Chryseobacterium*.

The other strain isolated from raw chicken, 5_R23647, clustered with *C. piscium*, *C. balustinum* and *C. scophthalmum* as its closest phylogenetic neighbours. This phylogeny's stability was supported by a bootstrap re-sampling value of 81%. It was obtained with the two other treeing methods albeit at 53% and 62% bootstrap re-sampling support values for the maximum parsimony analysis and the UPGMA algorithm, respectively. *Chryseobacterium piscium*, *C. balustinum* and *C. scophthalmum* were isolated from diseased and/or healthy fish (Mudarris *et al*., 1994; de Beer *et al*., 2005; Bernardet and Nakagawa, 2006).

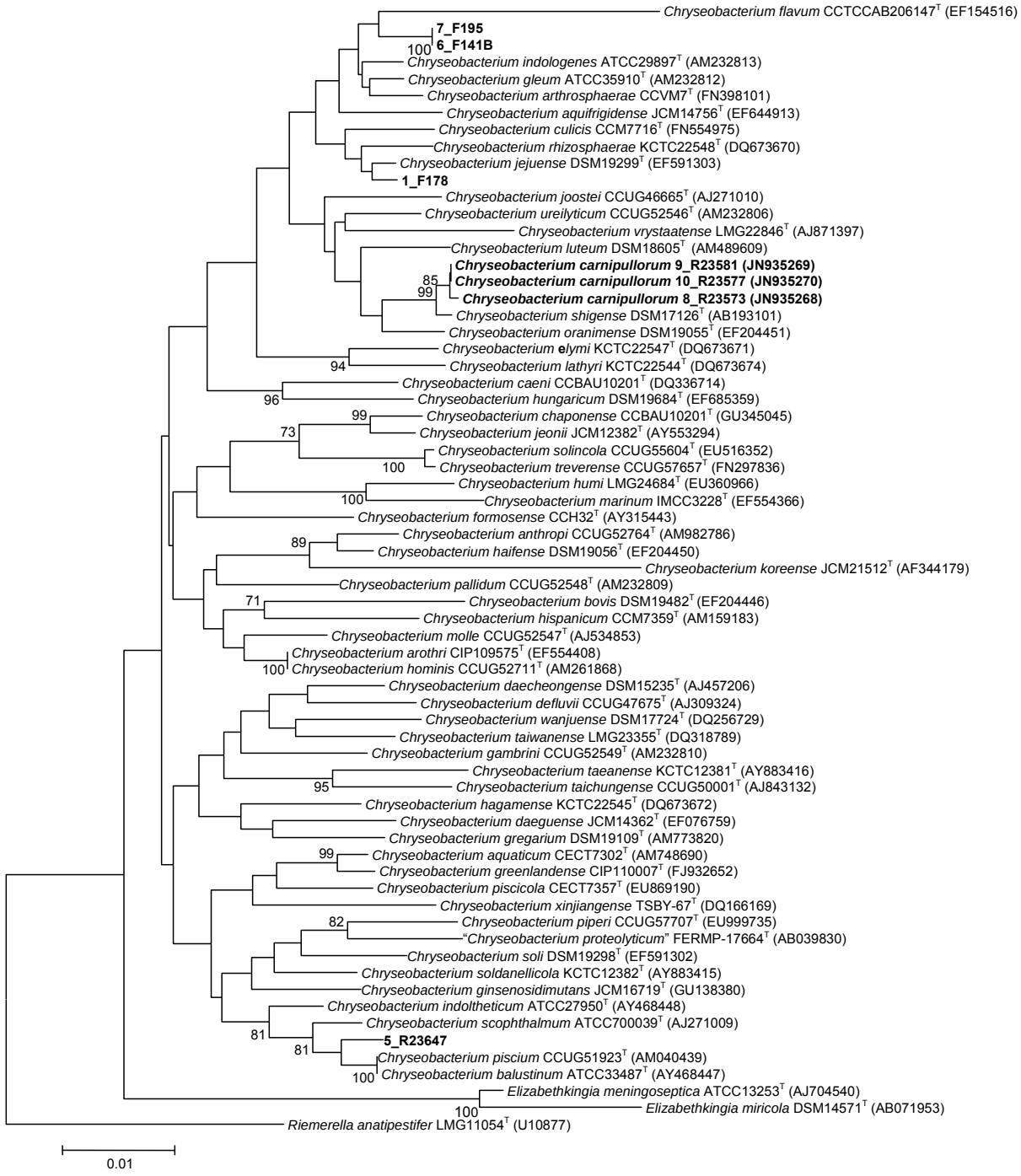


Figure 4.2. Phylogenetic analysis of strains 1_F178, 5_R23647, 6_F141B, 7_F195, 8_R23573, 9_R23581 and 10_R23577 and all currently recognised *Chryseobacterium* type species based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses). Multiple alignments were performed and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the neighbour-joining method in the MEGA version 4 software package (Tamura *et al*., 2007). Bootstrap values >70%, based on 1000 replications, are given as percentages at the branching points. Bar, 0.01 substitutions per nucleotide position.

Strain 1_F178 was isolated from chicken feathers from a second poultry-processing plant in Bloemfontein, South Africa. The plant disposes of most of its chicken feather waste by burying and strain 1_F178 was obtained from feathers that had been buried for 1 week (Chapter 3). Its nearest phylogenetic neighbour was *C. jejuense*. This phylogeny was moderately supported by a bootstrap re-sampling value 68%. However, it was also obtained using the other two methods. Strain 1_F178 formed a sub-cluster with *C. rhizosphaerae* and *C. culicis*. This sub-cluster comprised mostly of soil isolates and was also obtained using the other two treeing methods. *Chryseobacterium jejuense* was isolated from soil samples from Jeju, Republic of Korea (Weon *et al*., 2008). However, *C. culicis* was isolated from the midgut of the mosquito *Culex quinquefascitus* (Kämpfer *et al*., 2010b).

Strains 6_F141B and 7_F195 had *C. flavum* as their nearest phylogenetic neighbour. The two strains formed a tight cluster between themselves (100% bootstrap re-sampling support) indicating that they are likely to be strains belonging to a single species. This phylogeny was also obtained using the other tree-making methods with high bootstrap re-sampling support values of 99 and 100% for the maximum parsimony and the UPGMA methods respectively (Appendices I and II respectively). Both were isolated from chicken feathers at a chicken processing plant in Bloemfontein, South Africa. They formed a sub-line with intermediate bootstrap re-sampling support value (50%) which included the type strain of the type species, *C. gleum* F93^{T}. Even though this sub-line was obtained using the other two tree-making methods, it had mixed support by bootstrap re-sampling values of 20% and 78% for the maximum parsimony and the UPGMA methods, respectively. This clearly showed that the sub-line was not stable and that the tight cluster by the unknown strains was polyphyletic within the sub-line.

4.3.4. *Phenotypic differentiation: conventional tests*

The reference strains were selected on the basis of being the nearest phylogenetic neighbours or, in the case of *C. gleum*, of being the type species of the genus *Chryseobacterium* in accordance with the recommendations for phenotypic analysis by Tindall *et al*. (2010). In cases where the nearest phylogenetic neighbour was not

available, the type species was employed. The results for the seven isolates will now be discussed with emphasis on comparisons to reference strains that were found to be the closest phylogenetic neighbours of the strains in question. Comparison of the physiological and biochemical characteristics enabled differentiation of each isolate from the other isolates and from the reference strains.

Table 4.8 outlines the distinctive phenotypic characteristics of all the seven isolates and the nine reference strains used. All the strains were positive for production of catalase, oxidase, lecithinase and phosphatase. They also produced yellow colonies with flexirubin-type pigment and had a fruity odour. They hydrolysed esculin, casein, Tween 20, gelatine, 0.4% selinite, and starch. Hydrolysis of starch results for *Chryseobacterium piscium* and *C. scophthalmum* strains used in this study were at variance with literature (Bernardet *et al*., 2011). After three re-tests on different days, they remained strongly positive for starch hydrolysis after 24 h. All the strains were negative for Gram-reaction, motility, gluconate oxidation, H_2S production by the TSI method, Voges Proskauer test, growth on Simmon's Citrate Agar, arginine desimidase activity, fluorescence on King's medium, KCN tolerance, nitrite reduction, utilization of adonitol, L-arabinose, dulcitol and D-xylose. All strains were positive for indole production except *C. scophthalmum*. All strains hydrolysed Tween 20 except *C. piscium* and *C. balustinum*.

All the strains were positive for growth in 1 and 2% sodium chloride. Strains 10_R23577, *C. shigense*, 6_F141B, 7_F195, *C. indologenes*, 1_F178, *C. piscium* and *C. scophthalmum* grew in 3% NaCl while strains 8_R23573, 9_R23581, *C. ureilyticum*, *C. gleum*, 5_R23647 had delayed growth. *Chryseobacterium luteum*, *C. oranimense* and *C. balustinum* failed to grow in 3% NaCl. None of the isolates or reference strains were able to grow in 4 and 5% NaCl. *Chryseobacterium ureilyticum*, *C. gleum* and *C. indologenes* were negative for growth at 5 ^{o}C while the following strains showed weak growth: 8_R23573, 9_R23581, 10_R23577, *C. shigense, C. oranimense,* 6_F141B, 7_F195, *C. balustinum,* 1_F178, *C. piscium* and *C. scophthalmum*. *C. luteum* and 5_R23647 were positive for growth at 5 ^{o}C. All the strains grew well at 25 and 30 ^{o}C. Three strains grew at 37 ^{o}C (*C. oranimense*, *C. gleum* and *C. indologenes*) and the rest did not grow. None of the strains were able to grow at 42 ^{o}C except for *C. gleum*.

None of the strains were able to produce acid from 10% glucose except *C. shigense, C. ureilyticum*, *C. oranimense* and *C. gleum*. None of the strains were able to produce acid from 10% lactose except *C. shigense, C. oranimense* and 5_R23647. All strains were negative for the methyl red test except *C. shigense* and *C. oranimense*. None of strains were able to grow on Cetrimide Agar except *C. ureilyticum*, *C. indologenes* and 1_F178. All strains were able to grow on MacConkey Agar except *C. scophthalmum*. The other tests gave variable results as shown in Table 4.8.

4.3.4.1. *Isolates 8_R23573, 9_R23581 and 10_R23577*

Strains 8_R23573, 9_R23581 and 10_R23577 differed among themselves with regard to production of 3-ketolactose (Table 4.8). Only isolate 8_R23573 was able to produce 3-ketolactose. Hence isolates 9_R23581 and 10_R23577 could not be distinguished in this way from 8_R23573. The three strains were almost identical phenotypically indicating that they belonged to the same species.

The nearest phylogenetic neighbour of strains 8_R23573, 9_R23581 and 10_R23577, in terms of reference strains, was *C. shigense*. The strains could however be differentiated from *C. shigense* by a negative methyl red reaction, blood haemolysis tests, failure to produce acid from 10% glucose and 10% lactose, by their ability for assimilation-alkalinization of citrate in Christensen's citrate agar and inability to produce a brown pigment on tyrosine agar.

The strains were phenotypically also similar to *C. luteum* but they could be differentiated by their ability to assimilate and alkalinize citrate in Christensen's citrate agar; hydrolysis of tyrosine and ethanol, their capacity to reduce nitrate within 5 days and the absence of β–galactosidase displayed by their failure to utilise o-nitrophenyl-β-D-galactopyranoside (ONPG).

Table 4.8. Phenotypic characterisation of *Chryseobacterium* Isolates and closely related taxa.

Characteristic	8_R23573	9_R23581	10_R23577	*C. shigense* DSM 17126^T	*C. luteum* LMG23785^T	*C. ureilyticum* DSM18017^T	*C. oranimense* DSM19055^T	1_F178	*C. gleum* NCTC 11432^T	6_F141B	7_F195	*C. indologenes* LMG8337^T	5_R23647	*C. piscium* LMG23089^T	*C. balustinum* NCTC 11212^T	*C. scophthalmum* LMG 13028^T
Methyl red test	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Growth with 3% NaCl	w	w	+	+	-	w	-	+	w	+	+	+	w	+	-	+
Growth on:																
MacConkey agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+
Cetrimide agar	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-
Marine agar	-	w	-	w	-	+	+	+	+	+	+	+	+	+	-	+
Haemolysis of blood on Blood agar	-	-	-	+	-	-	-	+	-	+	+	+	+	+	+	+
Growth at (^oC):																
5	w	w	w	w	+	-	w	w	-	w	w	-	+	w	w	w
37	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-
42	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Acid production from:																
10% glucose	-	-	-	+	-	+	+	-	+	-	-	-	-	-	-	-
10% lactose	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-
Ethanol	+	+	+	+	-	-	+	-	-	-	-	-	+	+	+	+
Alkaline reaction on:																
Christensen's citrate agar	+	+	+	-	-	-	+	+	+	+	+	+	-	+	-	-
Production of:																

Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
3-Ketolactose	+	-	-	+	+	+	-	+	-	+	-	-	-	-	+	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	w	+	w	+	w	+	w	w
Ornithine decarboxylase	w	w	-	w	-	-	-	-	-	-	-	-	w	-	-	w
L-Phenylalanine deaminase	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
β -Galactosidase	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	+
Brown pigment on Tyrosine agar	-	-	-	+	-	+	-	-	-	-	-	+	+	-	+	+
Urease on Christensen's urea agar	-	-	-	-	-	+	-	+	+	+	-	+	-	+	-	+
Utilization of Malonate	w	w	w	w	-	w	-	w	-	w	w	+	w	-	-	w
Degradation of:																
Tween 20	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Tyrosine	+	+	+	+	-	+	+	-	-	-	-	-	w	-	+	+
Reduction of:																
Nitrate reduction	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+
Nitrite reduction	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Mol% G+C	36.9	36.7	36.6	36.6	ND	36.4	ND	ND	36-39	ND	ND	37-39	ND	34	35	33-35

All data are from this study. +, positive; -, negative; w, weakly positive. All strains were positive for: catalase, oxidase, production of yellow colonies with flexirubin-type pigment and a fruity odour, DNase, lecithinase, phosphatase; growth in the presence of 1 and 2% NaCl, growth at 25 and 30 ^{o}C, growth on MacConkey Agar, hydrolysis of esculin, casein, Tween 80, gelatine, 0.4% selinite, and starch. All strains were negative for: Gram-reaction, motility, growth in the presence of 4 and 5% NaCl, gluconate oxidation, H_2S production by the TSI method, Voges Proskauer test, growth on Simmon's Citrate Agar, arginine desimidase, fluorescence on King's medium, adonitol utilization, KCN tolerance, nitrite reduction, acid production from L-arabinose, dulcitol, D-xylose.

The strains could be differentiated from *C. gleum* by lack of growth at 37 and 42 ^{o}C; inability to produce acid from 10% glucose; inability to hydrolyse tyrosine and ethanol; lack of urease activity on Christensen's urea agar; inability to reduce nitrite and a negative ONPG test.

The strains could be differentiated from *C. ureilyticum* by their failure to grow on cetrimide agar; failure to utilise 10% glucose; ability to utilise citrate, inability to produce a brown pigment on tyrosine agar, hydrolysis of ethanol and lack of urease activity.

They could be distinguished from *C. oranimense* by their inability to produce and maintain stable acidic end products from glucose fermentation and to overcome the buffering capacity of the system as shown by negative methyl red tests; inability to grow at 37 ^{o}C, lack of acid production from 10% glucose and 10% lactose and a negative ONPG test.

The strains could be differentiated from *C. piscium* by their failure to haemolyse blood, their weak or no growth on marine agar, negative lysine decarboxylase test, ability to hydrolyse tyrosine and Tween 20 and lack of urease activity on Christensen's urea agar.

The strains could be differentiated from *C. balustinum* by their weak or strong growth in 3% NaCl, inability to haemolyse blood, produce a brown pigment on tyrosine agar; ability to produce phenylalanine deaminase and hydrolyse Tween 20.

The unknown species was distinguishable from *C. scophthalmum* by producing indole (Kovac's test) and their inability to haemolyse blood, lack of a brown pigmentation on tyrosine agar, lack of urease activity on Christensen's urea agar and a negative ONPG test.

4.3.4.2. *Isolates 6_F141B and 7_F195*

Reference strain *Chryseobacterium indologenes* had the highest 16S rRNA gene sequence similarity to isolate 6_F141B. It formed a tight cluster in the phylogenetic

tree with isolate 7_F195, *C. flavum*, *C. gleum* and *C. arthrosphaerae*. *Chryseobacterium indologenes* and *C. gleum* were the reference strains that were available. Isolate 6_F141B could be differentiated from *C. indologenes* since it was negative for growth on cetrimide agar, growth at 37 ^{o}C, production of brown pigment on tyrosine agar and nitrate reduction. It also differed in having the ability to produce 3-ketolactose and phenylalanine deaminase. In comparison to *C. gleum*, isolate 6_F141B did not grow at 37 and 42 ^{o}C; did not produce acid from 10% glucose; did not reduce nitrate and nitrite, and had a negative ONPG test. It had the ability to haemolyse blood and was able to produce 3-ketolactose. Isolates 6_F141b and 7_F195 were phenotypically very similar except for production of 3-ketolactose and urease activity which were positive for isolate 6_F141B and negative for isolate 7_F195.

Isolate 7_F195 could be distinguished from *C. indologenes* by its inability to grow on cetrimide agar; failure to grow at 37 ^{o}C; inability to produce a brown pigment on tyrosine agar; negative urease activity and inability to reduce nitrate and its ability to produce phenylalanine deaminase.

4.3.4.3. *Isolate 1_F178*

Isolate 1_F178 was most closely related to *C. jejuense* which was not available as reference strain and it was compared to *C. gleum* (Table 4.8). Isolate 1_F178 displayed the following phenotypic differences compared to *C. gleum*: It had the ability to grow on cetrimide agar, haemolyse blood; produce 3-ketolactose; did not grow at 37 and 42 ^{o}C; failed to produce acid from 10% glucose; did not reduce nitrite and the ONPG test was negative.

4.3.4.4. *Isolate 5_R23647*

Isolate 5_R23647 was most closely related to *C. piscium*. Its phenotypic profile was mostly similar to *C. piscium* (Table 4.8) except for negative alkalinization of citrate and urease activity, and its ability to produce acid in 10% lactose. Isolate 5_R23647 differed from *C. gleum* in that it was capable of haemolysing blood; growing at 5 ^{o}C, producing acid from 10% lactose; producing a brown pigment on tyrosine agar and

hydrolysing ethanol. It also differed in not growing at 37 and 42 ^{o}C; could not produce acid from 10% glucose; could not alkalinize citrate; showed no urease activity; could not reduce nitrite and had a negative ONPG test. In comparison to *C. balustinum*, isolate 5_R23647 was able to grow on marine agar, produce acid from 10% lactose; produce phenylalanine deaminase and hydrolyse Tween 20. Isolate 5_R23647 could be differentiated from *C. scophthalmum* by its negative ONPG and urease tests and it was positive for indole production.

4.3.5. *Phenotypic differentiation: BIOLOG Omnilog Gen III*

The BIOLOG Omnilog identification system is an advanced tool for identifying and characterizing organisms. It uses each microbe's ability to use particular carbon sources from a 71 substrate assay and its sensitivity to 23 inhibitory chemicals. Tetrazolium redox dyes are used to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals. There is increased respiration during incubation in the wells where cells can utilise the substrate or grow in the presence of inhibitors. The increased respiration leads to the reduction of the tetrazolium redox dyes, forming a purple colour. This allows the formation of a metabolic fingerprint (BIOLOG Inc., 2008). The fingerprint data is imported into the Omnilog data collection software, which searches the database and makes an identification call.

Table 4.9 shows the phenotypic identification of the seven isolates and nine reference strains using the BIOLOG Omnilog Gen III identification system. The SIM value is a critical identification call parameter and must range from 0.5 to 1.0 after at least 8 h of incubation for an identification to be made. Identification calls made after less than 8 h of incubation require a SIM value of 1.0. An identification is called after two consecutive readings where the SIM value is above 0.5.

The percentage of strains that were identified to species level was 56.25% (9/16) with SIM values ranging from 0.619 to 0.817 while 37.50% (6/16) were identified to genus level with SIM values ranging from 0.539 to 0.839 and 6.25% (1/16) could not be identified by the system since it had a low SIM value of 0.475.

Table 4.9. Phenotypic identification of the seven isolates and nine reference strains using the BIOLOG Omnilog Gen III identification system.

Strain	PROB*	SIM**	ID***
8_R23573	-	0.475	No ID
9_R23581	0.787	0.787	*C. scophthalmum*
10_R23577	0.669	0.669	*C. scophthalmum*
C. shigense DSM 17126^T	0.734	0.734	*C. scophthalmum*
C. luteum LMG23785^T	-	0.675	*Chryseobacterium* genus
C. ureilyticum DSM18017^T	0.623	0.623	*Flavobacterium tirrenicum/Chryseobacterium*
C. oranimense DSM19055^T	0.976	0.746	*C. gleum*/*indologenes*
C. gleum NCTC 11432^T	0.999	0.764	*C. gleum*/*indologenes*
6_F141B	0.676	0.676	*Flavobacterium tirrenicum/Chryseobacterium*
7_F195	0.734	0.553	*Flavobacterium tirrenicum /Chryseobacterium*
C. indologenes LMG8337^T	0.648	0.648	*C. gleum/indologenes*
1_F178	0.641	0.539	*Flavobacterium tirrenicum/Chryseobacterium*
5_R23647	-	0.839	*Chryseobacterium* genus
C. piscium LMG23089^T	0.928	0.817	*C. scophthalmum*
C. balustinum NCTC 11212^T	0.619	0.619	*C. balustinum*
C. scophthalmum LMG 13028^T	0.928	0.781	*C. scophthalmum*

*, confidence probability of a called ID; **, ID score value; ***, microbe identification

The Omnilog system was able to identify all the reference strains that are found in its data base. The similarity values of the reference strains ranged from 0.619 to 0.817 with a probability range of 0.619 to 0.999.

Even though isolates 8_R23573, 9_R23581 and 10_R23577 belonged to one group representing a single species, two strains, 9_R23581 and 10_R23577, were identified as *C. scophthalmum.* Isolate 8_R23573 could not be identified since its SIM was below 0.5.

Isolates 6_F141B, 7_F195 and 1_F178 were identified as *Flavobacterium tirrenicum/Chryseobacterium* with SIM values of 0.676, 0.553 and 0.539, respectively and isolate 5_R23674 was identified as *Chryseobacterium* genus with a SIM value of 0.839. The Omnilog system could not differentiate between *Flavobacterium tirrenicum* and *Chryseobacterium*. *Flavobacterium tirrenicum* has many phenotypic characteristics in common with *Chryseobacterium* such that prior to 1994, the then *Flavobacterium* genus contained, among others, *Flavobacterium tirrenicum* and species that were later transferred to the new genus *Chryseobacterium* (Vandamme *et al*., 1994). The paucity of *Chryseobacterium* species in the Omnilog data base reduced the options for comparing with many fingerprints and thus compromising the accuracy of the identifications.

4.3.6. *BIOLOG Omnilog Gen III phenotypic profiling*

Characteristics that differentiated the isolates using the BIOLOG Omnilog Gen III identification system are presented in Tables 4.10 to 4.14. This system was selected to characterise the seven isolates and nine reference strains.

The system was not only used to identify the strains since it currently has only five *Chryseobacterium* species (*gleum*/*indologenes*, *indoltheticum*, *balustinum* and *scophthalmum*) in its database compared to the 58 species that have been described to date, but mainly to characterize cellular functions. The Omnilog substrates were divided into the following substrate guilds: sugars (including hexose phosphates and miscellaneous substrates); amino acids; hexose acids; carboxylic

acids, esters and fatty acids; and inhibitory chemicals. The results will now be discussed with regard to these substrate categories.

Tables 4.10 to 4.14 show the results for different substrate guilds. All 16 strains studied were positive for pectin and tetrazolium blue. All strains were negative for: D-cellobiose, D-turanose, stachyose, α-D-lactose, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-neuraminic acid, D-sorbitol, D-arabitol, myo-inositol, D-aspartic acid, D-serine, L-galactonic acid lactone, D-gluconic acid, p-hydroxy-phenylacetic acid, methyl pyruvate, L-malic acid, bromo-succinic acid, α-hydroxy-butyric acid, α-keto-butyric acid, propionic acid, 8% NaCl, niaproof 4, vancomycin and nalidixic acid.

4.3.6.1. *Bacterial isolates 8_R23573, 9_R23581 and 10_R23577 from raw chicken portions*

Table 4.10 shows the utilization of substrates in the sugars guild (which includes hexose phosphates and miscellaneous substrates) by the seven unknown isolates as well as the nine reference strains. The three strains from raw chicken samples, 8_R23573, 9_R23581 and 10_R23577, produced nearly the same phenotypic fingerprint with the sugars. They could be differentiated from each other by the following: strain 9_R23581 was weakly positive with dextrin while 8_R23573 and 10_R23577 were negative; strain 9_R23581 was weakly positive with gentiobiose and D-mannose while 8_R23573 and 10_R23577 were positive; strain 8_R23573 was weakly positive with sucrose, while the other two were negative; strain 8_R23573 was weakly positive for 3-methyl glucose, D-fucose and L-rhamnose and the other two strains were negative. Strains 8_R23573 and 9_R23581 were weakly positive for D-fucose and strain 10_R23577 was negative.

Comparison of the three strains to the two nearest phylogenetic neighbours showed that *C. shigense* could be differentiated from the unknown strains due to its weakly positive utilization of D-raffinose, D-salicin, D-fructose, D-galactose, 3-methyl glucose, D-fucose and L-fucose. *Chryseobacterium luteum* could be differentiated because it was negative for gentiobiose, α-D-glucose and D-mannose for which the

Table 4.10. Sugars (including hexose phosphates and miscellaneous substrates) chemical guild differential characteristics of *Chryseobacterium* isolates and closely related taxa using the Biolog Omnilog Gen III system

Substrate	8_R23573	9_R23581	10_R23577	*C. shigense* DSM 17126^T	*C. luteum* LMG23785^T	*C. ureilyticum* DSM18017^T	*C. oranimense* DSM19055^T	1_F178	*C. gleum* NCTC 11432^T	6_F141B	7_F195	*C. indologenes* LMG8337^T	5_R23647	*C. piscium* LMG23089^T	*C. balustinum* NCTC 11212^T	*C. scophthalmum* LMG 13028^T
Dextrin	-	w	-	w	-	w	+	+	+	w	+	w	-	+	+	-
D-Maltose	-	-	-	-	-	-	w	w	+	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	-	-	w	-	+	-	-	-	-	-	-	w
Gentiobiose	+	w	+	w	-	+	+	+	+	+	+	+	+	+	+	+
Sucrose	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-
D-Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
β-Methyl-D-Glucoside	-	-	-	-	-	--	-	-	+	-	-	-	-	-	-	-
D-Salicin	-	-	-	w	-	-	+	-	+	-	-	-	-	-	-	-
N-Acetyl- D-Galactosamine	-	-	-	-	-	-	w	w	w	w	-	w	-	-	+	-
α-D-Glucose	w	w	w	w	-	+	+	+	+	+	+	+	+	+	-	+
D-Mannose	+	w	+	w	-	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	-	-	-	w	-	-	+	w	+	w	-	-	-	-	-	+
D-Galactose	-	-	-	w	-	-	w	w	w	w	+	w	-	-	+	-
3-Methyl Glucose	w	-	-	w	-	w	w	w	w	w	+	w	-	-	+	-
D-Fucose	w	-	-	w	-	w	w	w	w	+	+	+	-	-	+	-
L-Fucose	w	w	-	w	-	w	w	w	w	+	+	+	-	-	+	-
L-Rhamnose	w	-	-	-	-	-	w	w	-	w	+	w	+	-	+	+

Inosine	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
D-Mannitol	-	-	-	w	-	-	-	w	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
D-Fructose-6-Phosphate	w	-	-	w	-	w	w	w	+	+	+	+	-	-	+	-

Symbols: +, positive; -, negative; w, weakly positive. All data are from this study. All strains were negative for: D-cellobiose, D-turanose, stachyose, α-D-lactose, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-neuraminic acid, D-sorbitol, D-arabitol and myo-inositol.

three strains were either positive or weakly positive. The three strains could not utilize the hexose-phosphate sugars (D-glucose-6-phosphate and D-fructose-6-phosphate) except for strain 8_R23573 which was weakly positive with D-fructose-6-phosphate. The utilization of hexose-phosphates by the nearest phylogenetic neighbours reference strains was similar to that of the three unknown strains. Therefore utilization of hexose-phosphate could not differentiate between these taxa.

Table 4.11 shows the utilization of amino acids by the seven unknown isolates as well as the nine reference strains. Strain 8_R23573 was able to utilize the protein gelatine and L-glutamic acid but was weakly positive for L-aspartic acid utilization. Strain 9_R23581 utilized gelatine and had a delayed utilization of L-glutamic acid. Strain 10_R23577 could be differentiated from the other two strains since it was negative for all the amino acids tested. The amino acid profiles of the three strains compared to the two closest phylogenetic neighbours (*C. shigense* and *C. luteum*) were similar. Even though the amino acid profiles did not differentiate between the strains and their nearest neighbours, they suggested that the strains belong to the genus *Chryseobacterium*.

The hexose acids profiles for the strains were similar except for D-glucuronic acid which was weakly positive for strain 8_R23573 (Table 4.12). This profile was the same for *C. shigense* but *C. luteum* differed in that it had a negative D-glucuronic acid test. Reactions in the carboxylic acids, esters and fatty acids substrate guild was strain dependant and isolates could not be differentiated from the reference strains. The only exception was the utilization of acetic acid which was negative for *C. luteum* but positive for *C. shigense* and the three strains (Table 4.13).

In the chemical sensitivity substrates guild (Table 4.14), all three unknown strains from raw chicken (8_R23573, 9_R23581 and 10_R23577) and their two nearest phylogenetic neighbours (*C. shigense* and *C. luteum*) were able to grow at pH 6 but not pH 5. They mostly had delayed growth in the presence of 1% NaCl and they were all sensitive to 4 and 8% NaCl. *Chryseobacterium gleum* was also sensitive to 4 and 8% NaCl. Two out of the three strains as well as the reference strains were able to utilise 1% sodium lactate.

Table 4.11. Amino acids chemical guild differential characteristics of *Chryseobacterium* isolates and closely related taxa using the Biolog Omnilog Gen III system.

Substrate	8_R23573	9_R23581	10_R23577	*C. shigense* DSM 17126^T	*C. luteum* LMG23785^T	*C. ureilyticum* DSM18017^T	*C. oranimense* DSM19055^T	1_F178	*C. gleum* NCTC 11432^T	6_F141B	7_F195	*C. indologenes* LMG8337^T	5_R23647	*C. piscium* LMG23089^T	*C. balustinum* NCTC 11212^T	*C. scophthalmum* LMG 13028^T
Gelatin	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
Glycyl-L-Proline	-	-	-	-	-	-	-	w	+	-	-	-	-	-	-	+
L-Alanine	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
L-Arginine	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-
L-Aspartic acid	w	-	-	w	-	w	w	-	+	-	-	-	-	-	-	-
L-Glutamic Acid	+	w	-	w	-	w	+	+	+	+	+	w	-	w	-	+
L-Histidine	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
L-Serine	-	-	-	-	-	-	-	w	+	-	-	-	-	-	-	-

Symbols: +, positive; -, negative; w, weakly positive. All data are from this study. All strains were negative for D-aspartic acid and D-serine.

Table 4.12. Hexose acids chemical guild differential characteristics of *Chryseobacterium* isolates and closely related taxa using the Biolog Omnilog Gen III system.

Substrate	8_R23573	9_R23581	10_R23577	*C. shigense* DSM 17126^T	*C. luteum* LMG23785^T	*C. ureilyticum* DSM18017^T	*C. oranimense* DSM19055^T	1_F178	*C. gleum* NCTC 11432^T	6_F141B	7_F195	*C. indologenes* LMG8337^T	5_R23647	*C. piscium* LMG23089^T	*C. balustinum* NCTC 11212^T	*C. scophthalmum* LMG 13028^T
D-Galacturonic Acid	+	+	+	+	-	w	w	w	w	+	+	+	+	+	+	+
D-Glucuronic Acid	w	-	-	w	-	-	w	w	-	w	+	w	-	-	+	-
Glucuronamide	-	-	-	-	-	-	-	w	w	w	+	w	-	-	+	-
Mucic Acid	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Quinic Acid	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-

Symbols: +, positive; -, negative; w, weakly positive. All data are from this study. All strains were positive for pectin. All strains were negative for L-galactonic acid lactone and D-gluconic acid.

Table 4.13. Carboxylic acids, esters and fatty acids chemical guild differential characteristics of *Chryseobacterium* isolates and closely related taxa using the Biolog Omnilog Gen III system.

Substrate	8_R23573	9_R23581	10_R23577	*C. shigense* DSM 17126^T	*C. luteum* LMG23785^T	*C. ureilyticum* DSM18017^T	*C. oranimense* DSM19055^T	1_F178	*C. gleum* NCTC 11432^T	6_F141B	7_F195	*C. indologenes* LMG8337^T	5_R23647	*C. piscium* LMG23089^T	*C. balustinum* NCTC 11212^T	*C. scophthalmum* LMG 13028^T
D-Lactic Acid Methyl Ester	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Citric Acid	+	w	-	-	-	-	+	w	+	w	-	w	-	-	-	-
α-Keto-Glutaric Acid	-	-	-	-	-	-	-	w	w	-	-	-	-	-	-	-
D-Malic Acid	-	-	-	w	-	-	-	-	w	-	-	-	-	-	-	-
Tween 40	-	w	-	+	-	-	+	-	+	-	-	-	-	-	-	-
γ-Amino-Butyric Acid	-	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoacetic Acid	+	w	+	w	-	+	+	+	+	+	+	+	+	+	-	+
Acetic Acid	+	+	w	+	-	+	+	+	+	+	+	+	+	+	-	+
Formic Acid	-	w	-	-	-	w	-	-	+	-	+	w	-	-	-	-

Symbols: +, positive; -, negative; w, weakly positive. All data are from this study. All strains were negative for p-hydroxy-phenylacetic acid, methyl pyruvate, L-malic acid, bromo-succinic acid, α-hydroxy-butyric acid, α-keto-butyric acid and propionic acid.

Table 4.14. Inhibitory substances chemical guild differential characteristics of *Chryseobacterium* isolates and closely related taxa using the Biolog Omnilog Gen III system.

Substrate	8_R23573	9_R23581	10_R23577	*C. shigense* DSM 17126^T	*C. luteum* LMG23785^T	*C. ureilyticum* DSM18017^T	*C. oranimense* DSM19055^T	1_F178	*C. gleum* NCTC 11432^T	6_F141B	7_F195	*C. indologenes* LMG8337^T	5_R23647	*C. piscium* LMG23089^T	*C. balustinum* NCTC 11212^T	*C. scophthalmum* LMG 13028^T
pH 6	+	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 5	-	-	-	-	-	-	-	-	+	-	w	-	-	-	-	-
1% NaCl	w	w	w	w	+	+	+	+	+	+	+	+	+	+	+	+
4% NaCl	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
1% Sodium Lactate	+	+	-	w	+	+	+	+	+	+	+	+	+	+	w	+
Fusidic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
D-Serine	+	w	-	w	-	+	-	+	+	+	+	+	-	-	+	-
Troleandomycin	+	w	-	w	-	+	+	+	w	+	+	+	+	-	-	-
Rifamycin SV	-	-	-	w	-	-	+	w	+	-	-	+	-	-	-	-
Minocycline	-	-	-	-	-	+	-	+	+	w	+	+	-	-	-	-
Lincomycin	+	w	w	w	-	+	+	+	+	+	+	+	-	+	-	+
Guanidine HCl	-	w	-	w	-	+	-	w	+	+	+	+	-	w	-	+
Tetrazolium Violet	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+
Lithium Chloride	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Potassium Tellurite	-	-	-	w	-	+	-	-	+	+	+	+	-	-	-	-
Aztreonam	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium Butyrate	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Sodium Bromate	-	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-

Symbols: +, positive; -, negative; w, weakly positive. All data are from this study. All strains were positive for tetrazolium blue. All strains were negative for 8% NaCl, niaproof 4, vancomycin and nalidixic acid.

The three strains had nearly the same susceptibilities than *C. shigense* and *C. luteum* to lithium chloride, potassium tellurite, sodium butyrate and sodium bromate. None of the five strains were sensitive to aztreonam. *Chryseobacterium indologenes* could be differentiated from the strains by its lack of susceptibility to lithium chloride, potassium tellurite, aztreonam and sodium butyrate.

All the taxa displayed strong reducing power since they reduced tetrazolium violet and tetrazolium blue. *Chryseobacterium shigense* was partially susceptible to D-serine, treandomycin, rifamycin SV, lincomycin and guanidine HCl while *C. luteum* was susceptible to all. Results for the species from raw chicken were strain dependent making differentiation of the new species impossible. However, the strains could be differentiated from *C. gleum* since it was not susceptible to minocycline while all three strains from raw chicken were susceptible.

Overall, strain 9_R23581 had the most central characteristics and it was chosen as the type strain of the unknown species.

4.3.6.2. *Isolates 6_F141B and 7_F195 from chicken feather waste*

Isolates 6_F141B and 7_F195 from chicken feather waste were compared to two reference strains, *C. indologenes* and *C. gleum* (type species) as their nearest phylogenetic neighbours. In the sugar utilization guild, the two strains were differentiated from each other by a weakly positive *N*-acetyl-D-galactosamine and D-fructose reaction for isolate 6_F141B while isolate 7_F195 was negative (Table 4.10). Isolate 6_F141B's results were similar to the reference strain *C. indologenes* except for D-fructose which was weakly positive for the former but negative for the latter.

The isolates could be differentiated from *C. gleum* because they could not utilise β–methyl-D-glucoside, D-salicin and glycerol. The rest of the pattern for utilization of the sugars was the same suggesting that the taxa belonged to the same genus. The two hexose-phosphates had the same results for both the isolates and the reference strains except for *C. gleum* which was positive for D-glucose-6-phosphate.

The two isolates had identical results for amino acid tests (Table 4.11). They varied from the *C. gleum* reference culture by failing to utilise D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-histidine, L-pyroglutamic acid and L-serine.

Utilization of hexose acids by both chicken feather waste isolates 6_F141B and 7_F195 was similar (Table 4.12). All strains were able to utilise pectin, D-galacturonic acid, D-glucuronic acid, and glucuronamide. This pattern was similar to that of *C. indologenes* but differed from *C. gleum* because of *C. gleum*'s failure to use L-galactonic acid lactone and D-glucuronic acid, but it was able to utilise mucic acid, quinic acid and D-saccharic acid while the other three taxa were not.

With regard to the carboxylic acids, esters and fatty acids substrate guild (Table 4.13), the two chicken feather waste isolates 6_F141B and 7_F195 and both *C. gleum* and *C. indologenes* were all negative for p-hydroxy-phenylacetic acid, methyl pyruvate, L-lactic acid, L-malic acid, bromo-succinic acid, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, α-ketobutyric acid and propionic acid. They were all able to utilise acetoacetic acid and acetic acid. The two isolates differed from each other only in the utilization of citric acid (weakly positive for isolate 6_F141B and negative for isolate 7_F195) and formic acid (negative for isolate 6_F141B and positive for isolate 7_F195). The isolates could be differentiated from *C. gleum* in that they were unable to utilise D-lactic acid methyl ester and Tween 40. Isolate 6_F141B was similar to *C. indologenes* except for formic acid which the former was unable to utilise compared to the latter which weakly utilised this substrate. The main difference between isolate 7_F195 and *C. indologenes* was the inability of isolate 7_F195 to utilize citric acid.

Table 4.14 shows the chemical susceptibilities of isolates 6_F141B and 7_F195 and reference strains. All the four taxa grew well at pH 6 but there were variations at pH 5. Isolate 6_F141B and *C. indologenes* were negative compared to *C. gleum* while isolate 7_F195 was weakly positive. All the strains except *C. indologenes* tolerated 1% NaCl, but were sensitive to 4% NaCl. All the strains

were sensitive to 8% NaCl, were able to utilise 1% sodium lactate, and had strong reducing power since both tetrazolium violet and tetrazolium blue were positive. All four taxa were resistant to fusic acid, D-serine, troleandomycin, lincomycin and guanidine HCl. Both isolates were susceptible to rifamycin SV while both reference strains were resistant.

Taking all 94 substrates into account, strains 6_F141B and 7_F195 shared more phenotypic characteristics with *C. indologenes* than with *C. gleum*.

4.3.6.3 *Chicken feather waste isolate 1_F178*

Isolate 1_F178 was compared to *C. gleum*. The strains had the same characteristics for the sugars substrates guild except for D-trehalose, β-methyl-D-glucose, D-salicin and inosine utilization which was positive for *C. gleum* but negative for isolate 1_F178. The hexose-phosphate, D-glucose-6-phosphate, was utilized by *C. gleum* and not by isolate 1_F178 (Table 4.10).

Amino acids utilization of isolate 1_F178 differed from *C. gleum* in that it was unable to utilise D-serine, L-alanine, L-aspartic acid, L-pyroglutamic acid and L-serine (Table 4.11). It was also negative for L-arginine and L-histidine while *C. gleum* had intermediate reactions.

The hexose acids that could differentiate isolate 1_F178 from *C. gleum* were mucic acid which was negative for isolate 1_F178. Quinic acid and D-saccharic acid gave intermediate reactions with *C. gleum* while isolate 1_F178 was negative (Table 4.12). Utilization of the rest of the hexose acids was similar for all the strains.

Isolate 1_F178 was distinct from *C. gleum* in the carboxylic acids, esters and fatty acids substrates group in that it was negative for D-lactic acid methyl ester, Tween 40 and formic acid utilization (Table 4.13). The other substrates were almost the same.

Sensitivity tests showed that isolate 1_F178 was more sensitive to low pH than *C. gleum* (Table 4.14). Both strains grew at pH 6 but only *C. gleum* grew at pH 5. The other characteristic differentiating this strain from *C. gleum* was its sensitivity to potassium tellurite while *C. gleum* was not. The rest of the sensitivity tests had similar results.

4.3.6.4. *Isolate 5_R23647 from raw chicken*

In the 16S rRNA phylogenetic tree, isolate 5_R23647 was phylogenetically related to *C. piscium*, *C. balustinum* and *C. scophthalmum*. Isolate 5_R23647 could be differentiated from *C. piscium* by its inability to utilise dextrin while *C. piscium* was positive (Table 4.10). Another characteristic which differed in the sugars substrates group was that isolate 5_R23647 utilised L-rhamnose while *C. piscium* could not.

Isolate 5_R23647 in comparison to *C. balustinum* differed in that it was negative for dextrin, D-melibiose and N-acetyl-D-galactosamine while *C. balustinum* was positive. This isolate also differed in that it was able to utilise α-D-glucose while *C. balustinum* was not. The rest of the substrates in this group gave similar reactions for both strains.

Isolate 5_R23647 differed from *C. scophthalmum* in that it was not capable of utilizing D-fructose. All the other substrates in this group gave similar reactions for both strains.

Hexose phosphates could not distinguish between isolate 5_R23647 and *C. piscium* and *C. scophthalmum*. The isolate could be differentiated from *C. balustinum* by its failure to utilise D-fructose-6-phosphate.

Amino acid utilization profiles were almost similar for isolate 5_R23647, *C. piscium*, *C. balustinum* and *C. scophthalmum* (Table 4.11). The only differences were that isolate 5_R23647, *C. piscium* and *C. scophthalmum* were able to utilise gelatine while *C. balustinum* was not. Isolate 5_R23647 and *C. balustinum* were

negative for L-glutamic acid utilization while *C. piscium* gave an intermediate reaction and *C. scophthalmum* gave a positive reaction.

Hexose acid utilization (Table 4.12) could not differentiate among isolate 5_R23647, *C. piscium* and *C. scophthalmum*. Isolate 5_R23647 could be differentiated from *C. balustinum* in that it was unable to utilise D-glucuronic acid and glucuronamide.

Isolate 5_R23647 was distinct from *C. balustinum* in the carboxylic acids, esters and fatty acids substrates guild in that it was negative for L-lactic acid while *C. balustinum* gave a positive reaction (Table 4.13). Isolate 5_R23647 could also be differentiated from *C. balustinum* in that it was able to utilise acetoacetic acid and acetic acid while *C. balustinum* was not. Isolate 5_R23647 could not be differentiated from either *C. piscium* or *C. scophthalmum* in this substrate group since they all shared the same characteristics.

Susceptibility tests revealed that isolate 5_R23647 was resistant to troleandomycin (Table 4.14). This characteristic was the only one that could differentiate the isolate from *C. piscium*, *C. balustinum* and *C. scophthalmum* since all these reference strains were susceptible. The other substrates gave similar or varied results.

An overview of all the isolates in this study showed that they could be placed into four groups: Group 1, 8_R23573, 9_R23581 and 10_R23577; Group 2, 6_F141B and 7_F195; Group 3, 1_F178 and Group 4, 5_R23647. The members of these groups most probably belong to four new species since they could not, using the tests above, be equated with any known species. This was supported by the 16S rRNA data that also indicated that the isolates could possibly represent four new species. However, these isolates need further polyphasic investigations to meet the requirements for new species status.

4.4. Conclusions

From the phylogenetic part of the study, the following conclusions are drawn:

i. Optimisation of the PCR procedure using an annealing temperature of 53 ^{o}C eliminated all non-specific binding.

ii. 48.28% (14/29) of the isolates investigated in this study were successfully identified as belonging to the genus *Chryseobacterium* using 16S rRNA gene sequencing.

iii. Comparative 16S rRNA gene sequence analysis among the seven strains under study revealed that only strains 8_R23573, 9_R23581 and 10_R23577 isolated from raw chicken had a high phylogenetic relatedness (99% similarity) while the rest of the strains (1_F178, 6_F141B and 7_F195) from chicken feather waste and 5_R23647 from raw chicken had lower phylogenetic relatedness (≤97% similarity).

iv. Three of the four raw chicken isolates, 8_R23573, 9_R23581 and 10_R23577, exhibited the highest levels of 16S rRNA gene sequence similarity with *C. shigense* (99.22, 99.78 and 99.71%) and the isolates from chicken feather waste, 6_F141B and 7_F195, and 1_F178, showed highest levels of 16S rRNA gene sequence similarity with *C. indologenes* and *C. jejuense* (98.04–98.12% and 98.99% respectively). The fourth raw chicken isolate, 5_R23647, was highly similar to *C. piscium* (98.85%).

v. The phylogenies of the seven isolates obtained using the neighbour-joining tree-making method were obtained using two other methods (maximum parsimony and UPGMA) indicating their stability.

From phenotypic characterization, the following conclusions are drawn:

i. The BIOLOG Omnilog Gen III identification system was able to identify all reference strains that are in its data base but its main limitation was the small database for the genus *Chryseobacterium.*

ii. Both the conventional tests and the BIOLOG Omnilog Gen III identification system were able to differentiate between the unknown strains and the reference strains.

iii. The seven unidentified strains could be regarded as belonging in four new species represented by the following strains: 1_F178; 5_R23647; 6_F141B and 7_F195; and 8_R23573, 9_R23581 and 10_R23577. The phenotypic consistency supported earlier findings from phylogenetic analysis.

CHAPTER 5

POLYPHASIC TAXONOMIC STUDY OF *Chryseobacterium* ISOLATES FROM RAW CHICKEN AND THE DESCRIPTION OF *Chryseobacterium carnipullorum* sp. nov.

ABSTRACT

Three Gram-negative, rod-shaped, non-spore-forming, non-motile, oxidase positive, yellow pigmented and aerobic bacterial isolates designated 8_R23573, 9_R23581 and 10_R23577 were isolated from raw chicken in a broiler processing plant. They were investigated using a polyphasic taxonomic approach to determine their exact taxonomic identities. The most abundant quinone was menaquinone MK-6 and the predominant cellular fatty acids were 15:0 iso, iso 17:1 ω9c, 17:0 iso 3OH and summed feature 3, consisting of 16:1 ω7c or 15:0 iso 2OH, supported the affiliation of the strains to the genus *Chryseobacterium.* The DNA base compositions of the strains were 36.9, 36.7 and 36.6 mol% G+C respectively. This further confirmed the affiliation of the strains to the genus *Chryseobacterium* whose DNA G+C content ranges from 29 to 39 mol% G+C (Bernardet *et al*., 2011). The DNA-DNA hybridization results gave relatedness values of more than 81% among the three strains and less than 57% similarity between the strains and the two nearest phylogenetic neighbours *Chryseobacterium shigense* (DSM 17126^T) and *Chryseobacterium luteum* (LMG23785^T). This provided evidence for a novel species when recommendations of a threshold value of 70% DNA-DNA similarity for the delineation of bacterial species were considered (Wayne *et al.*, 1987). Comparisons involving results of phylogenetic analysis, DNA-DNA hybridization, chemotaxonomic and biochemical tests enabled the genotypic and phenotypic differentiation of the strains from the next most closely related *Chryseobacterium* species with validly published names. On the basis of the data from this polyphasic study, the three strains represent a novel species of the genus *Chryseobacterium* for which the name *Chryseobacterium carnipullorum* sp. nov. is proposed. The type strain is 9_R23581 (= DSMxxxxx^T, = LMGzzzzz^T, = CCUGyyyyy^T).

5.1. Introduction

The genus *Chryseobacterium* belongs to the family *Flavobacteriaceae* (Bernardet *et al*., 2006). It was described by Vandamme *et al.* (1994a) to accommodate six species formerly classified within the genus *Flavobacterium*, namely *Chryseobacterium balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum*. The type species is *C. gleum*. It includes the psychrotolerant and proteolytic spoilage microorganisms that are widely distributed in food sources such as milk, fish, meat and poultry (Hugo *et al*., 2003; de Beer *et al*., 2006).

In 2006 the genus *Chryseobacterium* consisted of only 11 recognised species including "*C. proteolyticum*" (although the name has not been validly published), but excluding *Chryseobacterium meningosepticum* and *C. miricola.* The latter two species have recently been transferred to the new genus *Elizabethkingia* under the new epithets *Elizabethkingia meningoseptica* and *E. miricola* (Kim *et al*., 2005a). Since then the genus has undergone significant and rapid expansion and to date it comprises of 58 species and more continue to be described (Euzéby, 2012b). This can be ascribed to the readily available and improved phenotypic, chemotaxonomic and molecular identification methods used in the polyphasic taxonomic approach. Polyphasic taxonomy is an old concept first mooted by Colwell (1970). Many levels of information such as phenotypic, genomic and ecological levels are extracted from a non-homogeneous system and analysed to delineate consensus taxonomic groups.

Nine novel species that were validly published in 2011 are: *C. elymi, C. hagamense, C. lathyri* and *C. rhizosphaerae* isolated from the rhizosphere of coastal sand dune plants (Cho *et al*., 2011); *C. chaponense*, isolated from farmed Atlantic salmon (Kämpfer *et al*., 2011); *C. ginsenosidimutans*, isolated from soil of a *Rhus vernicifera*-cultivated field (Im *et al*., 2011); *C. piperi* isolated from a fresh water creek (Strahan *et al*., 2011); *C. xinjiangense* isolated from alpine permafrost (Zhao *et al*., 2011) and *C. yonginense* isolated from a mesotrophic artificial lake (Joung and Joh, 2011).

Members of the family *Flavobacteriaceae* occur in a wide range of habitats including freshwater (Manz *et al*., 1999; Brummer *et al*., 2000), marine environments (Kirchman, 2002), polar regions (Ravenschlag and Dworkin, 2001), industrial sources (Whitley and Bailey, 2000), biofilms (Kirchman, 2002) as well as soil and food sources (Bernardet, 2011). The genus *Chryseobacterium* is one of the 10 genera of the family *Flavobacteriaceae* which is associated with food. Other food associated genera are *Bergeyella*, *Empedobacter*, *Flagellimonas*, *Flavobacterium* (type genus), *Myroides*, *Salegentibacter*, *Tenacibaculum*, *Vitellibacter* and *Weeksella* (Hugo and Jooste, 2003; 2012). A number of studies have isolated flavobacteria from meat where they are regarded as food spoilers (Forsythe, 2000). As the taxonomy and history of flavobacteria evolved, most of the food spoilage flavobacteria were reclassified in the new genus *Chryseobacterium* (Bernardet *et al*., 1996).

In the previous chapter, seven isolates were characterized phenotypically and their phylogenies were determined based on 16S rRNA gene sequence analysis. These isolates formed four distinct groups (group 1: 1_F178; group 2: 5_R23647; group 3: 6_F141B and 7_F195; and group 4: 8_R23573, 9_R23581 and 10_R23577) that were not affiliated to any of the currently recognised *Chryseobacterium* species. All the isolates require further polyphasic investigations to have full evidence for description as new species.

The first aim of this study was to perform a polyphasic study on some of the isolates studied in Chapter 4. Three isolates, 8_R23573, 9_R23581 and 10_R23577, which clustered tightly together and apparently belonged to the same species, were selected for further evaluation. Several techniques were employed to collect relevant information needed to decide the exact taxonomic identities of the strains. Techniques used in this part of the study included the determination of DNA base composition by HPLC, spectroscopic DNA-DNA hybridization, fatty acid and quinone analysis by TLC. The second aim was to describe the new species taking into account previous phylogenetic and phenotypic findings (Chapter 4).

5.2. Materials and methods

5.2.1. *Cultures used and their maintenance*

The isolates used in this part of the study include three isolates from raw chicken namely, 8_R23573, 9_R23581 and 10_R23577, obtained from a previous study (de Beer, 2005). They were isolated from raw chicken in a poultry processing plant in Bloemfontein, the Free State Province, South Africa. The reference strains used in this study, *Chryseobacterium shigense* DSM 17126^T, *C. luteum* LMG23785^T and *C. gleum* NCTC 11432^T, were procured from international culture collections (DSMZ, LMG and NCTC) and they were preserved and cultured as described previously (Chapter 4). They were selected as reference strains because they were the nearest phylogenetic neighbours to the three isolates referred to above.

5.2.2. *Analyses of fatty acids and quinones*

Analyses of fatty acids and respiratory quinones were carried out by the DSMZ Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany.

5.2.2.1 *Fatty acid analysis sample preparation*

Cellular fatty acid analysis samples were prepared by streaking pure colonies onto Nutrient Agar slants and incubating at 25 ^oC for 24 h.

5.2.2.2. *Determination of fatty acids*

The samples were prepared using the standard method according to Kuykendall *et al*. (1988) and MIDI Inc. (2001). Identification was done using an Agilent 6890N gas chromatograph and version 6.1 of the MIDI Inc. Sherlock Microbial Identification System software.

5.2.2.3. *Determination of quinones*

Quinones analysis samples were prepared by freeze-drying to get 200 mg of each isolate. Organisms were grown on Nutrient Agar slants at 25 ^{o}C for 18 h. The slants were flooded with freeze-drying medium and the growth was scraped off and suspended in the medium. The suspension (0.5 ml) was added to freeze-drying vials and freeze dried using a Labconco Freezone (model 7934031) freeze dryer according to manufacturer's instructions.

5.2.2.4. *Extraction of respiratory lipoquinones*

Respiratory lipoquinones were extracted from 100 mg of the freeze dried cell material using the two stage method described by Tindall (1990a; 1990b).

5.2.2.5. *Analysis of respiratory lipoquinones*

Respiratory lipoquinones were separated into their different classes such as menaquinones and ubiquinones by thin layer chromatography on silica gel (Macherey-Nagel Art. No. 805 023), using hexane:*tert*-butylmethylether (9:1 v/v) as solvent. UV absorbing bands corresponding to the different quinone classes (e.g. menaquinones or ubiquinones) were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm x 125 mm, 3 µm, RP18) using methanol:heptane 9:1 (v/v) as the eluant. Respiratory lipoquinones were detected at 269 nm.

5.2.3. *DNA base composition*

5.2.3.1. *Sample preparation*

Wet biomass (3 g of each isolate) was required for both DNA-DNA hybridization (DDH) and DNA base composition analyses. Freeze-dried cultures were resuscitated in 10 ml of Nutrient Broth and incubated at 25 ^{o}C for 48 h. The cultures were streaked on Nutrient Agar and incubated at 25 ^{o}C for 4 8h. The colonies that developed were checked for purity. To grow the cell mass of the isolates, a pure colony from each plate was inoculated into a fresh 10 ml volume

of Nutrient Broth and incubated for 48 h at 25 ^{o}C. The resulting culture was inoculated into 200 ml of Nutrient Broth in shake flasks and incubated for 24 h. The cells were harvested by centrifugation at 10 000 rpm for 15 min in 50 ml Falcon tubes. The cells were washed with 0.01 M phosphate buffer pH 7.0, weighed off and preserved in isopropanol:water (1:1 v/v).

5.2.3.2. *Determination of G+C content of DNA*

The determination of the G+C content of DNA by HPLC was carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. DNA was isolated by cell disruption in a French pressure cell. Purification of the DNA on hydroapatite was done according to the procedure of Cashion *et al*. (1977). The DNA was degraded by hydrolysing with P1 nuclease and the nucleotides dephosphorylized with bovine alkaline phosphatase according to Mesbah *et al*. (1989). The resulting deoxyribonucleosides were analysed by HPLC (Shimadzu Corporation, Japan). It consisted of the following modules: LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-20A automatic sample injector and a SPD-20A UV spectrophotometric detector. Chromatograms were analysed using the CLARITY (version 2.4.1.93) software package (DataApex Ltd., Czech Republic).

The analytical column used was a VYDAC 201SP54, C18, 5 µm (250 x 4.6 mm) column equipped with a guard column 201GD54H (VYDAC, Hesperia, CA92345, USA). Chromatographic conditions were adapted from Tamaoka and Komagata (1984) as follows: a temperature of 45 ^{o}C, 10 µl sample, solvent 0.3 M $(NH_4)H_2PO_4$/acetonitrile, 40:1 (v/v), pH 4.4, and 1.3 ml/min. The reference DNA used was calibrated with non-methylated Lambda-DNA (Sigma), GC-content 49.858 mol% (Mesbah *et al*., 1989) and the following 3 DNAs for which complete genome sequences were published (http://ergo.integratedgenomics.com/GOLD/): *Bacillus subtilis* DSM402 (43.518 mol% G+C), *Xanthomonas campestris* pv. c*ampestris* DSM 3586^{T} (65.069 mol% G+C) and *Streptomyces violaceoruber* DSM40783 (72.119 mol% G+C). The GC content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah *et al*. (1989).

5.2.4. *Spectroscopic DNA-DNA hybridization (DDH)*

5.2.4.1. *Sample preparation*

The samples were prepared as described above for the determination of DNA base composition.

5.2.4.2. *Determination of DDH values*

The DDH values were determined spectroscopically as follows: Cells were disrupted in a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al*. (1977). DDH was carried out as described by De Ley *et al*. (1970) under consideration of the modifications described by Huss *et al*. (1983) using a model Cary 100Bio uv/vis-spectrophotometer equipped with a peltier-thermostatted 6x6 multicell charger and a temperature controller with in-situ temperature probe (Varian).

5.3. Results and discussion

5.3.1. *Cellular fatty acids*

Cellular fatty acid (CFA) profiles of strains 8_R23573, 9_R23581 and 10_R23577 and the type strains of their two nearest phylogenetic neighbours, *C. shigense* and *C. luteum*, are shown in Table 5.1. The most predominant fatty acid for the unknown strains was 15:0 iso ranging from 42.35 to 43.54%. The second most predominant fatty acid was 17:1 ω9c, ranging from 18.90% to 20.14%. The third most predominant fatty acid was 17:0 iso 3OH ranging from 15.67 to 15.97%.

Table 5.1. Cellular fatty acid profiles of isolates 8_R23573, 9_R23581 and 10_R23577 and phylogenetically related type strains in the genus *Chryseobacterium.*

Fatty acid (%)	**8_R23573**	**9_R23581**	**10_R23577**	*C. shigense* **DSM 17126^T**	*C. luteum* **LMG23785^T**
13:0 iso	1.68	1.76	1.57	1.12	1.22
ECL 13.565	0.55	Tr	Tr	0.53	Tr
15:0 iso	43.54	42.79	42.35	38.50	41.50
15:0 ante iso	2.48	2.60	2.56	3.70	0.63
15:0 3OH	2.58	2.65	2.59	2.82	3.02
16:0	0.57	0.53	0.55	0.94	ND
16:0 iso	Tr	Tr	Tr	Tr	0.98
16:0 3OH	0.87	0.84	0.83	0.83	1.14
16:0 iso 3OH	0.65	0.70	0.71	0.54	Tr
ECL 16.582	1.15	1.14	1.20	1.42	1.35
17:0 iso	0.50	Tr	Tr	0.80	0.93
17:0 2OH	0.67	0.60	0.71	1.57	Tr
17:0 iso 3OH	15.97	15.67	15.68	17.84	19.15
Iso 17:1 ω9c	18.90	19.73	20.14	21.04	17.69
18:1 ω5c	1.15	1.22	1.15	0.83	0.59
Summed feature 3	7.24	7.54	7.42	6.27	10.31
Summed feature 4	0.61	0.62	0.68	0.57	0.29

All data are from this study. Values are percentages of the total. Fatty acids that amounted to <0.5% in all strains are not shown. ECL, equivalent chain length; Tr, Trace (<0.5%); ND, not detected; Summed feature 3, 16:1 ω7c or 15:0 iso 2OH or both; Summed feature 4, 15:0 iso 2OH or 16:1 ω7t or both.

The strains also contained, inter alia, moderate to small amounts of summed feature 3 constituting 16:1 ω7c or 15:0 iso 2OH (7.24 to 7.54%); 15:0 3OH (2.65 to 2.59%); 15:0 ante iso (2.48 to 2.60%); ECL 13.565 (1.57 to 1.76%); ECL 16.582 (1.14 to 1.20%) and/or 18:1 ω5c (1.15 to 1.22%).

Whole cell fatty acid compositions provide useful taxonomic information at the genus and species level for the taxon *Chryseobacterium* (Bernardet *et al*., 2002). *Chryseobacterium* can be differentiated from other members of the clade such as *Elizabethkingia*, *Empedobacter*, *Riemerella* and *Bergeyella* since they all display distinct fatty acid compositions (Hugo *et al*., 1999; Bernardet *et al*., 2006; 2011). Hence, fatty acid profiles of the family *Flavobacteriaceae* are mostly chemotaxonomic markers at the genus level (Bernardet and Nakagawa, 2006; Bernardet *et al*., 2011).

From Table 5.1, it is evident that the presence of large amounts of 15:0 iso, 17:1 ω9c and 17:0 iso 3OH fatty acids supported the affiliation of the three unknown strains to the genus *Chryseobacterium*. Furthermore, the CFA profiles of the strains were very similar to each other supporting the suggestion deduced from phylogenetic analysis that the three strains were affiliated to one species. However, these CFA profiles could not differentiate the strains at species level from the type strains of the two closest phylogenetic neighbours used as reference strains in this part of the study. Minor differences found among the profiles of the unknown strains compared to the reference standards were in the proportions of the fatty acids. These findings were similar to Hugo *et al*. (1999) who noted that whole cell fatty acid analysis profiles could not differentiate between *Chryseobacterium* strains.

5.3.2. *DNA base composition*

The DNA base composition of strains 8_R23573, 9_R23581 and 10_R23577 were 36.9, 36.7 and 36.6 mol% G+C respectively (Table 5.2). This provided further evidence of the affiliation of the strains to the genus *Chryseobacterium* whose DNA G+C content ranges from 29 to 39 mol% G+C (Bernardet *et al*., 2011).

Table 5.2. DNA-DNA hybridization results and DNA base compositions of strains 8_R23573, 9_R23581 and 10_R23577, and the type species of the closest phylogenetic neighbours in the genus *Chryseobacterium.*

	Mol% G+C	**8-R23573**	**9_R23581**	**10_R23577**	*C. shigense* **DSM 17126^T**	*C. luteum* **LMG8337^T**
8-R23573	36.9	ND				
9_R23581	36.7	87.1 86.5	ND			
10_R23577	36.6	81.4 85.9	87.2 78.8	ND		
C. shigense **DSM 17126^T**	36.6*	42.1 43.8	51.3 56.1	45.3 43.0	ND	
C. luteum **LMG8337^T**	ND**	35.7 27.4	23.4 25.6	32.2 28.2	14.0** 10.6**	ND

Data were from this study except otherwise stated. ND, Not determined.
*Data from Shimomura *et al*. (2005); **Data from Behrendt *et al*. (2007)

Strain 10_R23577 had the same mol% G+C value of 36.6 compared to one of the two nearest phylogenetic neighbours, *C. shigense*. The DNA base composition of the second nearest phylogenetic neighbour, *C. luteum*, was not determined when the species was described (Behrendt *et al*., 2007).

The major respiratory quinone for all three unknown strains was MK-6 ranging from 97 to 98% while MK-5 was the minor respiratory quinone ranging from 2 to 3% (Table 5.3). This was in accordance with all members of the genus *Chryseobacterium* (Bernardet *et al*., 2011) as well as members of the family *Flavobacteriaceae* (Bernardet, 2011).

Table 5.3. Respiratory quinones of strains 8_R23573, 9_R23581 and 10_R23577.

Strain	MK-5 (%)	MK-6 (%)
8-R23573	3	97
9_R23581	3	97
10_R23577	2	98

5.3.3. *DNA-DNA hybridization*

The DNA-DNA hybridization matrix for strains 8_R23573, 9_R23581 and 10_R23577 and the type strains of the two most closely related species of the genus *Chryseobacterium* is shown in Table 5.2. The DNA-DNA reassociation values among the strains ranged from 78.8 to 87.2%. These values indicated that the unknown bacterial strains from raw chicken were affiliated to the same species when recommendations by Wayne *et al*. (1987) and Rosseló-Mora (2006) of strain affiliation to a species are considered. They recommended a threshold value of 70% DNA-DNA similarity between strains for novel species delineation. Values below 70% imply that strains belong to distinct genomic species, and conversely, values above 70% indicate same species status.

The DNA-DNA reassociation values for strain 8_R23573 with *C. shigense* and *C. luteum* were 42.1% (reciprocal 43.8%) and 35.7% (reciprocal 27.4%), respectively. DNA-DNA hybridizations between strains 9_R23581 with *C. shigense* and *C. luteum* gave relatedness values of 51.3% (reciprocal 56.1%) and 23.4% (reciprocal 25.6%) respectively. The DNA-DNA reassociation values between strain 10_R23577 with *C. shigense* and *C. luteum* were 45.3% (reciprocal 43.0%), and 32.2% (reciprocal 28.2%), respectively. *Chryseobacterium shigense* and *C. luteum* gave DNA-DNA relatedness values of 14% (reciprocal 10.6%; Behrendt *et al*., 2007). All these values clearly confirmed that the unknown strains from raw chicken belong to a distinct novel genomic species when recommendations by Wayne *et al*. (1987) are considered.

5.3.4. *Description of Chryseobacterium carnipullorum sp. nov.*

Chryseobacterium carnipullorum (*car.ni.pul.lor'um.* L. n. *carno carnis* flesh; L. n. *pullus* a young animal, especially chicken; L. gen. n. *carnipullorum* of the flesh of chicken).

The strains are Gram-staining negative rods, non-spore forming, non-motile, non-gliding cells that are approximately 0.85 µm in length and 0.39 µm in width and have rounded ends. Colonies on Nutrient Agar are smooth, shiny with a butyraceous consistency and produce a deep yellow, non-diffusible, non-fluorescent, flexirubin-type pigment. They give off a fruity odour. Strains are aerobic, growing weakly at 5 ^{o}C after 10 days, but not at 37 and 42 ^{o}C. Optimal growth is at 25 to 30 ^{o}C. At 25 ^{o}C, 48 h, all strains grow on MacConkey Agar and have delayed growth on Christensen's citrate Agar, but not on Cetrimide Agar and Simmon's citrate Agar while growth on Marine Agar is strain dependent (1/3 positive). Colonies are not haemolytic on Blood Agar and do not produce acid from 10% glucose or 10% lactose. Strains are capable of growth in Nutrient Broth containing 1, 2 and 3% NaCl but not in 4, 5, and 8% NaCl.

All strains produce oxidase, catalase, phosphatase, DNase, lecithinase, indole, phenylalanine deaminase but not urease (Christensen's urea Agar) and β-galactosidase (ONPG test). Most strains (2/3) are negative for 3-ketolactose activity. Reaction on Triple Sugar Iron Agar (Oxoid CM277) is alkaline and no hydrogen sulphide is produced. Weak malonate utilization occurs.

The following substrates are hydrolysed namely: ethanol, casein, Tween 20, Tween 80, esculin, gelatine, 0.4% selenite, tyrosine (but brown pigmentation is not produced) and starch. Strains do not produce acid from 1% adonitol, L-arabinose, dulcitol and D-xylose. They are all sensitive to KCN at 0.0075% (w/v). Cells grow at pH 6 but not at pH 5. They grow in 1% NaCl but not in 4 and 8% NaCl.

The strains do not reduce nitrite but are positive for nitrate reduction. They are negative for gluconate oxidation, methyl red and Voges Proskauer tests.

The ability of the strains to utilise 95 carbon sources in the BIOLOG Gen II system, was tested. The strains utilised succinic acid mono-methyl ester, acetic acid, α-ketovaleric acid, L-asparagine and L-glutamic acid. Utilisation of propionic acid and glucuronamide is weak and strain dependent.

With the BIOLOG Omnilog Gen III system, strains were tested for their ability to utilise 71 carbon sources and their susceptibility to 23 inhibitory chemicals. The following substrates were hydrolysed: gentiobiose, D-mannose, gelatine, pectin, D-galacturonic acid, acetoacetic acid and acetic acid. Utilization of the following substrates is strain dependent: L-aspartic acid, L-glutamic acid, D-glucuronic acid, citric acid, Tween 40, γ-amino butyric acid and formic acid.

Strains are susceptible to fusidic acid, rifamycin, minocycline, niaproof, vancomycin and nalidixic acid, lithium chloride, potassium tellurite, sodium butyrate and sodium bromate but not to lincomycin and aztreonam. Strains have high reducing power to tetrazolium violet and tetrazolium blue.

The major fatty acids are 15:0 iso, 17:1 ω9c, 17:0 iso 3OH, summed feature 3 constituting 16:1 ω7c or 15:0 iso 2OH. The DNA G+C content of strain 9_R23581 is 36.7 mol% and that of the other strains ranges from 36.6 to 36.9 mol%.

The type strain is 9_R23581 and together with the other two strains (8_R23573 and 10_R23577) was isolated from raw chicken in a poultry processing plant in Bloemfontein, the Free State province, South Africa.

5.4. Conclusions

The following conclusions were drawn:

i. The cellular fatty acids for the isolates showed large amounts of 15:0 iso, 17:1 ω9c and 17:0 iso 3OH which was consistent with species affiliated to the genus *Chryseobacterium*.

ii. The major respiratory quinone was MK-6 which is in agreement with members of the genus *Chryseobacterium*.

iii. The DNA base composition ranged from 36.6 to 36.9 mol% G+C further confirming affiliation of the strains to the genus *Chryseobacterium* which has a DNA base composition range of 29 to 39 mol% G+C.

iv. DNA-DNA hybridization (DDH) values among the strains confirmed that they belonged to the same species. Moreover, this species had low DDH reassociation rates (27.4 to 56.1%) with type strains of the two nearest phylogenetic neighbours, *C. luteum* and *C. shigense*. This confirmed that the three strains are affiliated to a novel species.

v. A novel species with the proposed name, *Chryseobacterium carnipullorum*, was described. It consists of three strains 8_R23573, 9_R23581 and 10_R23577. Strain 9_R23581 had the most central characteristics and was chosen as the type strain.

Chapter 6

Phenotype microarray characterization of *Chryseobacterium carnipullorum* 9_R23581^T

Abstract

The BIOLOG Omnilog Phenotype MicroArray (PM) technology was developed recently in an attempt to give a global view of cellular phenotypes (phenomics). It consists of 20 panels with 96 wells each giving a total of 1920 assays. A novel species, *Chryseobacterium carnipullorum* 9_R23581^T, its nearest phylogenetic neighbour, *C. shigense* DSM 17126^T, and the type species for the genus *Chryseobacterium*, *C. gleum* NCTC 11432^T, were evaluated using the PM system. *Chryseobacterium carnipullorum* 9_R23581^T differed from *C. shigense* DSM 17126^T by 1.9% (37/1920). The organism differed from *C. gleum* NCTC 11432^T by 1.2% (23/1920). The phenotypes that differed were in the utilisation of antifungals, alcohols, amides, amino acids, carbohydrates, carboxylic acids, polymers, lipophilic chelators, esters, nitrite, cephalosporins and antibiotics. The phenotypes gained by *C. carnipullorum* 9_R23581^T indicate its high potential to spoil foods rich in carbohydrates, carboxylic acids and amino acids. Its ability to degrade substrates such as Y-cyclodextrin, pectin, gelatine, laminarin and mannan allows for the organism's enzymes to be potentially applied in biotechnological innovations involving polymer hydrolysis. This is the first time that global phenotyping using PM technology has been applied to *Chryseobacterium* species.

6.1. Introduction

The Phenotype MicroArrays (PMs) is a new technology for high-throughput simultaneous testing of a large number of cellular phenotypes. Bochner (1989) was the first to propose a technology for global (cell-wide perspective) analysis of cellular phenotypes using plates for high-throughput assays. Global cellular analyses provide data that will enable full understanding and description of a cell as a system. Global phenotypic analysis is critical in bacterial taxonomy since it allows more in-depth description and differentiation of species. It also augments the data generated by the polyphasic study in the previous chapter.

The first analytical tool developed to study cellular components in greater depth was the two-dimensional protein gel electrophoresis method for studying cellular proteins (O'Farrell, 1975). This enabled the analysis, in a single experiment, of levels and diversity of most proteins in a cell and it ultimately became the foundation of proteomics. Fodor *et al*. (1993) pioneered the second technology for nucleic acid analysis (genomics). The third technology that predicts the content or activities of cell metabolites led to the systems biology model known as metabolomics. This ultimately led to phenomics (Bochner, 2009).

The observable characteristics of cells (including "molecular phenotypes") are referred to as phenotypes. Growth phenotypes define how fast a bacterium will grow thereby allowing microbiologists to describe and differentiate cells (Bochner, 2009). In the past, phenotypes were measured one at a time. Bochner and co-workers developed a standard set of nearly 2000 assays that could be used productively with a wide range of bacterial species (Bochner *et al.,* 2001; Bochner, 2003).

The general outlook of a 20-panel PM system is shown in Figure 6.1. The assays are performed in 100 µl cultures in 20 x 96-well culture plates (PM1–20) making a total of 1920 phenotypic assays. The set consists of about 200 assays of C-source metabolism, 400 assays of N-source metabolism, 100 assays of biosynthetic pathways, 100 assays of ion effects and osmolarity, 100 assays of pH effects and pH control with deaminases and decarboxylases, and 1000 assays of chemical sensitivity. There are 240 diverse chemicals in the chemical sensitivity assays, each at four different concentrations (Bochner, 2009).

The BIOLOG Omnilog instrument, the Omnilog Combo System, reads and records colour changes in PM assays. A colour CCD camera reads the plates and provides quantitative and kinetic information on the response of the cells to the PMs. The BIOLOG technology tests cellular phenotypes using cell respiration as a reporter system (Bochner *et al*., 2001).

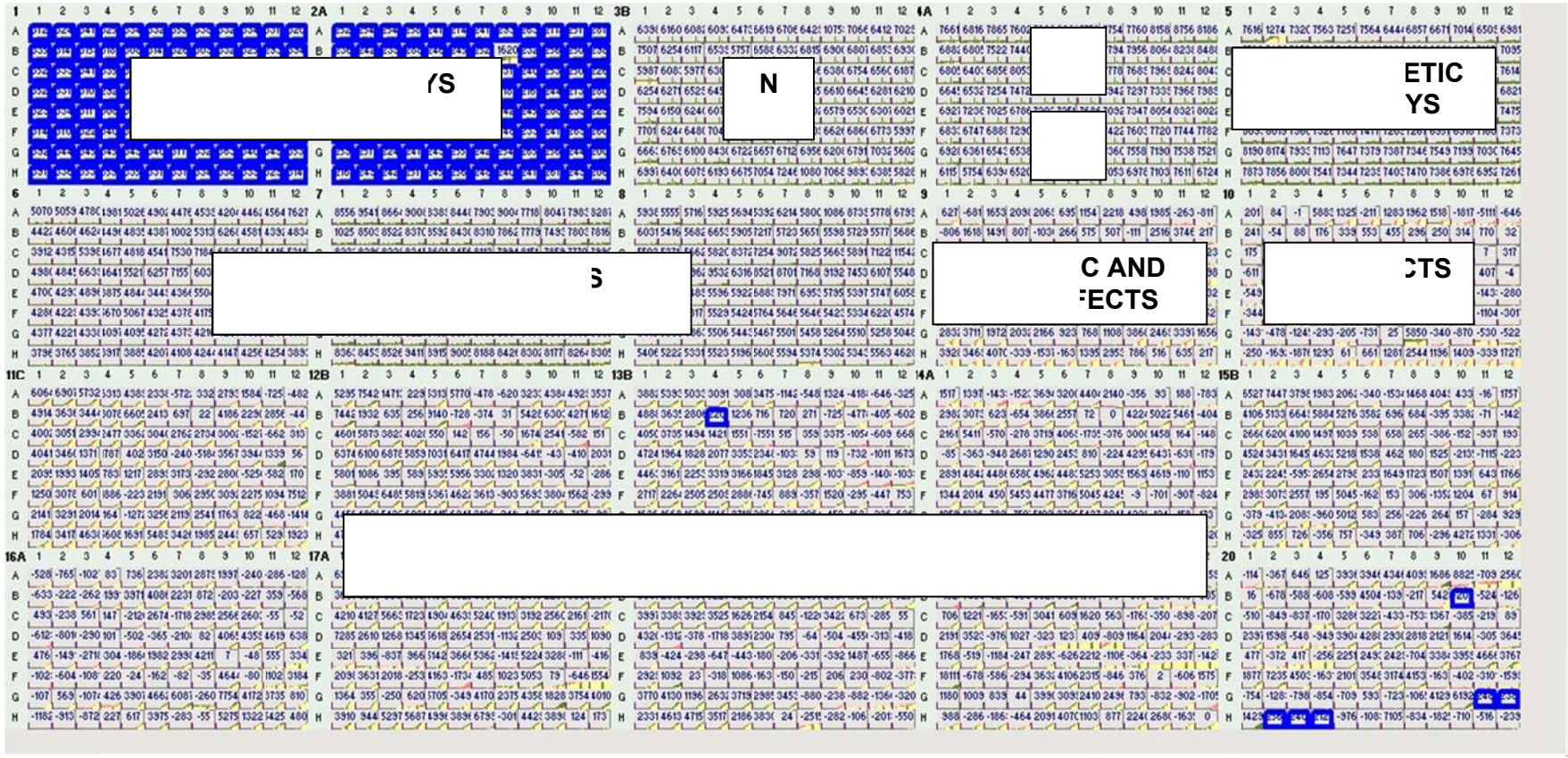


Fig. 6.1. Outline of a 20-panel, 1920 assays, Phenotype MicroArray system.

The assay uses a tetrazolium dye (mostly tetrazolium violet) to colorimetrically detect respiration in cells with the colour of the dye changing from colourless to purple. This dye reduction is essentially irreversible under physiological conditions leading to its accumulation over hours, amplifying the signal and integrating the amount of respiration over time.

This has some advantages such as the ease of monitoring and quantification of the colour change. The colour change is very sensitive and highly reproducible. Cell respiration can occur independently of cell growth and measurement of phenotypes that do not lead to growth is possible (Bochner *et al*., 2001).

The Omnilog Combo System is a versatile tool that is rapidly gaining in popularity among researchers. The main use of PM technology is the study of gene function. A direct way to assay gene function is to examine cells with knockouts of genes and see how the gene loss affects the phenotypes of the cells (BIOLOG Inc., 2001). Other uses of the technology include studying pathogenicity and epidemiology (Morales *et al*., 2005; 2006); the importance of metabolic and temperature signals in environmental sensing and pathogenesis (Mekalanos, 1992); studying cell transformations microscopic and macroscopic morphology; understanding the diversity of bacteria in terms of general phenotypic and culture properties; improving industrial bioprocesses; studying systems biology, taxonomy, bacterial identifications, microbial ecology, and evolution (Bochner, 2008).

In taxonomy, the subset of PM1, PM2 and PM9, provides a useful and broadly applicable set of almost 300 tests by which taxonomists can compare most of the fast-growing bacterial species with respect to categorizing them by the C-sources they utilise and the inorganic ions they are or are not compatible with (Bochner, 2009). In this part of the study, the novel organism, *Chryseobacterium carnipullorum* 9_R23581^{T}, was subjected to the BIOLOG Omnilog Combo System PM analysis and

compared to its closest phylogenetic neighbour, *C. shigense* DSM 17126^T and the type species, *C. gleum* NCTC 11432^T to get an indication of potential food spoilage characteristics and broader potential applications of the new species. This is the first time that any *Chryseobacterium* species has been phenotyped globally using the BIOLOG Omnilog PMs technology.

6.2. Materials and Methods

The test organism was *Chryseobacterium carnipullorum* 9_R23581^T and the reference strains used were *C. shigense* DSM 17126^T and *C. gleum* NCTC 11432^T.

The PM assays were carried out according to the manufacturer's (BIOLOG Inc.) instructions. The methods followed were "PM procedures for *E. coli* and other GN bacteria." The incubation temperature used was 25 ^oC for 24 h. All the panels, PM1 to 20, were tested.

The data was analysed using the RetroSpect Trending and Tracking® software version 2.1.1. A 20-panel comparison was done using the area under the curve parameter.

6.3. Results and discussion

6.3.1. *Phenotypic differentiation*

Table 6.1 shows the results of a 20-panel comparison of phenotypes naturally gained and lost (Table 6.2) by *C. carnipullorum* 9_R23581^T compared to *C. shigense* DSM 17126^T. Panels 1, 2A, 13B and 20 had some differences which were mostly minor.

Table 6.1. Phenotypes gained (faster growth/increased resistance) by *C. carnipullorum* 9_R23581^T compared to *C. shigense* DSM 17126^T.

Plate Type	Wells	Chemical	Importance
PM 20	H02, H03, H04	Patulin	antifungal
PM 02-A	H10	2,3-Butanediol	C-Source
PM 02-A	G01	Acetamide	C-source
PM 02-A	G02	L-Alaninamide	C-source
PM 02-A	H06	sec-Butylamine	C-source
PM 01	B12	L-Glutamic acid	C-source
PM 01	F01	Gly-Asp	C-source
PM 01	G01	Gly-Glu	C-source
PM 01	H01	Gly-Pro	C-source
PM 02-A	G06	L-Histidine	C-source
PM 02-A	G08	Hydroxy-L-Proline	C-source
PM 02-A	G09	L-Isoleucine	C-source
PM 02-A	G10	L-Leucine	C-source
PM 02-A	H04	L-Valine	C-source
PM 01	D12	Uridine	C-source
PM 02-A	B04	Amygdalin	C-source
PM 02-A	B10	i-Erythritol	C-source
PM 02-A	B12	3-0-β-D-Galactopyranosyl-D-Arabinose	C-source
PM 02-A	C10	α-Methyl-D-Mannoside	C-source
PM 01	G07	Acetoacetic acid	C-source
PM 02-A	D11	d-Amino Valeric acid	C-source
PM 02-A	D12	Butyric acid	C-source
PM 02-A	E02	Caproic acid	C-source
PM 02-A	E10	α-Keto-Valeric acid	C-source
PM 02-A	F11	D-Tartaric acid	C-source
PM 02-A	F12	L-Tartaric acid	C-source
PM 02-A	A02	Chondroitin Sulfate C	C-source
PM 02-A	A03	α-Cyclodextrin	C-source
PM 02-A	A04	β-Cyclodextrin	C-source
PM 02-A	A06	Dextrin	C-source
PM 02-A	A08	Glycogen	C-source
PM 02-A	A09	Inulin	C-source
PM 02-A	A10	Laminarin	C-source
PM 02-A	A11	Mannan	C-source
PM 02-A	A12	Pectin	C-source
PM 20	G11, G12	8-Hydroxyquinoline	chelator, lipophilic

Table 6.2. Phenotypes lost (slower growth/sensitivity) by *C. carnipullorum* 9_R23581^T compared to *C. shigense* DSM 17126^T.

Plate Type	Wells	Chemical	Importance
PM 20	B10	Tetrazolium Violet	respiration

The majority of the differences however were from PM1 and PM2A. The differences were mainly due to faster utilization of the substrates by *C. carnipullorum* 9_R23581^T compared to *C. shigense* DSM 17126^T. Those nutrients that are utilised at a faster rate are the organism's preferred nutrients (Lei, 2008). *Chryseobacterium carnipullorum* 9_R23581^T showed a total of 36 different phenotypes that it acquired, while only one phenotype (tetrazolium violet) showed a slower reaction compared to *C. shigense* DSM 17126^T.

Chryseobacterium carnipullorum 9_R23581^{T}showed increased resistance to the antifungal, patulin. Therefore, the phenotypes that differed between these two organisms were 1.9% (37/1920).

Chryseobacterium carnipullorum 9_R23581^T grew faster in the presence of each of the three amides, glucuronamide, acetamide and L-alaninamide. It also grew faster in wells containing the following: the amino acids, L-glutamic acid, Gly-Asp; Gly-Glu, Gly-Pro, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine and L-valine; the carbohydrates, uridine, amygdaline, i-erythritol, 3-0-β-D-galactopyranosyl-D-arabinose and α-methyl-D-mannoside; the carboxylic acids, acetoacetic acid, δ-amino valeric acid, butyric acid, caproic acid, α-keto-valeric acid, D-tartaric acid, L-tartaric acid; the polymers, chondroitin sulphate C, α-cyclodextrin, dextrin, glycogen, inulin, laminarin, mannan and pectin. The lipophilic chelator, 8-hydroxyquinoline, also resulted in faster growth for *C. carnipullorum* 9_R23581^T.

The results of a 20-panel comparison of phenotypes gained and lost by *C. carnipullorum* 9_R23581^T compared to *C. gleum* NCTC^T is shown in Tables 6.3 and 6.4 respectively.

Chryseobacterium carnipullorum 9_R23581^T had a total of 22 phenotypes which were different compared to *C. gleum* NCTC 11432^T. Sensitivity of the former to spiramycin was higher while its resistance to patulin was also higher.

The overall phenotypic differences between the two organisms amounted to 1.2% (23/1920). The existence of numerous bacterial species which colonised various niches in the world indicates the adaptability of the microorganisms to evolve and live in them (Bochner, 2009).

6.3.2. *Significance and potential applications of substrate utilization by C. carnipullorum 9_R23581^T*

The significance of the genus *Chryseobacterium* strains in the food environment includes its association with food spoilage. Members of the family *Flavobacteriaceae* have been linked to food deterioration but information on its role and incidence is difficult to obtain mainly due to the history of faulty classification or reclassification of these organisms. Until recently, literature with regard to food spoilage caused by these psychrotrophic bacteria still referred to this group as flavobacteria or *Flavobacterium*, or CDC Group IIb organisms. This reference could have included those that were reclassified and are now known as *Chryseobacterium* (de Beer, 2005; Bernardet *et al*., 2011).

In this study carbohydrates, carboxylic acids and amino acids were, by far, the most utilized substrate by *C. carnipullorum* 9_R23581^T and will now be discussed with respect to the metabolites produced and their impact on food quality.

Table 6.3. Phenotypes gained (faster growth/increased resistance) by *C. carnipullorum* 9_R23581^T compared to *C. gleum* NCTC 11432^T.

Plate Type	Wells	Chemical	Importance
PM 20	H03, H04	Patulin	antifungal
PM 02-A	H12	3-Hydroxy-2-butanone	C-source
PM 02-A	G02	L-Alaninamide	C-source
PM 01	B12	L-Glutamic acid	C-source
PM 01	D01	L-Asparagine	C-source
PM 01	E01	L-Glutamine	C-source
PM 01	F01	Gly-Asp	C-source
PM 01	G01	Gly-Glu	C-source
PM 01	H01	Gly-Pro	C-source
PM 01	D11	Sucrose	C-source
PM 02-A	B04	Amygdalin	C-source
PM 01	D06	α-Ketoglutaric acid	C-source
PM 01	G09	Mono-Methylsuccinate	C-source
PM 01	H10	D-Galacturonic acid	C-source
PM 02-A	F01	D-Lactic acid Methyl Ester	C-source
PM 02-A	A02	Chondroitin Sulfate C	C-source
PM 02-A	A07	Gelatin	C-source
PM 02-A	A09	Inulin	C-source
PM 02-A	A12	Pectin	C-source
PM 20	G12	8-Hydroxyquinoline	chelator, lipophilic
PM 09	H08	20mM Sodium Nitrite	toxicity, nitrite
PM 17-A	G12	Cefoperazone	wall, cephalosporin

Table 6.4. Phenotypes lost (slower growth) by *C. carnipullorum* 9_R23581^T compared to *C. gleum* NCTC 11432^T.

Plate Type	Wells	Chemical	Importance
PM 12-B	H03	Spiramycin	protein synthesis, 50S ribosomal subunit, macrolide

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in their sensory characteristics. Spoilt foods may be safe to eat if they do not cause illness because there are no pathogens or toxins present, but changes in texture, smell, taste or appearance cause them to be rejected depending on the individual's preferences, ethnic origin and family background (Garbutt, 1997; Madigan *et al*., 2000).

Carbohydrates were the most utilised substrates. Sixty-seven carbohydrate substrates were utilized by *C. carnipullorum* 9_R23581^T. If carbohydrates are available, they are usually preferred by bacteria over other energy-yielding nutrients (Mountney and Gould, 1988). Microbial fermentation of sugars can lead to souring and butyric spoilage defects. Metabolic products of carbohydrate utilization include organic acids, alcohols, carbon dioxide, hydrogen and water (Ayers *et al*., 1980; Banwart, 1989). The random splitting of glycosidic bonds results in textural defects due to softening and liquefaction (Chesson, 1980).

The second most utilized substrates were the carboxylic acids. The organism was able to metabolise 59 of these substrates. The carboxylic acids are weak organic acids containing oxygen (Ebbing and Garmon, 1999). Bacteria can oxidise most of these acids to carbohydrates, causing the medium to become more alkaline. Complete oxidation of carboxylic acids to carbon dioxide and water can occur under aerobic conditions. However, they may be partially oxidized to simpler acids or to products similar to those produced from sugars (Frazier, 1988). The simpler acids may cause sour tastes and α-amino acids may lead to bitter tastes in foods (Coultate, 1984).

Chryseobacterium carnipullorum 9_R23581^T was able to utilise 30 amino acid monomers and dimers. These were the third most utilized class of substrates. The oxidation or reduction of amino acids produces two organic acids, one molecule each of carbon dioxide, ammonia and hydrogen while the incomplete metabolism of amino acids can result in putrescence (Ayers

et al., 1980; Banwart, 1989). Hydrogen sulphide liberated from amino acids can lead to sulphide spoilage. The formation of ammonia from amino acids in meat and milk can result in alkalinization (Coultate, 1984).

6.3.3. *Potential applications*

It was found that *C. carnipullorum* 9_R23581^T was able to degrade 11 biopolymers including Y-cyclodextrin. Its degradation yields maltooligomers, such as maltooctaose, which can be applied in preparative carbohydrate chemistry as the carbohydrate moieties of artificial antigens. They can also be converted to suitable derivatized forms for use as chemical diagnostic agents (Jodal *et al*., 1984).

C. carnipullorum 9_R23581^T also has the potential of playing an important role in biopolymer degradation in (ultra)oligotrophic freshwater environments. *Flavobacterium johnsoniae* strain A3 was reported to especially use biopolymers at µg/l levels. Its growth in amylopectin, xyloglucan, gelatine, laminarin, and maltose or fructose at 0 to 200 µg/l resulted in oligosaccharides or oligopeptides as the main degradation products (Sack *et al*., 2011).

C. carnipullorum 9_R23581^T was able to degrade mannan faster than *C. gleum* NCTC 11432^T. Microbial mannases have become biotechnologically important since they target the hydrolysis of complex polysaccharides of plant tissues into simple molecules such as manno-oligosaccharides and mannases. The role of mannases in the paper and pulp industries is well established and recently they have found broader applications in food and stockfeed technology, coffee extraction, oil drilling and detergent industries. Bacterial mannases have an advantage over plant mannases in that they are mostly extracellular and can act in a wide range of pH and temperature (Dhawan *et al*., 2008).

6.4. Conclusions

Chryseobacterium carnipullorum 9_R23581^T, *C. shigense* DSM 17126^T and *C. gleum* NCTC 11432^T were successfully phenotyped globally using the BIOLOG Omnilog PMs technology. This is the first time that this technology has been applied to the genus *Chryseobacterium. Chryseobacterium carnipullorum* 9_R23581^T differed from *C. shigense* DSM 17126^T by 1.9% (37/1920) with differences in the following substrate classes: an antifungal, an alcohol, amides, amino acids, carbohydrates, polymers and a lipophilic chelator. It also differed from *C. gleum* NCTC 11432^T by 1.2% (23/1920). The substrate classes that differed were an antifungal, an alcohol, amides, amino acids, carbohydrates, polymers, a lipophilic chelator, an ester, nitrite, cephalosporin and an antibiotic. Global phenotypic analysis is critical in taxonomy since it allows more in-depth description and differentiation of species.

Chryseobacterium carnipullorum 9_R23581^T has the potential to spoil foods since it mostly utilized commonly found nutrients such as carbohydrates, carboxylic acids and amino acids whose metabolites lead to, inter alia, souring, butyric spoilage defects, alkalinization, bitter tastes and sulphide spoilage.

Enzymes which are produced by *C. carnipullorum* 9_R23581^T, can degrade a high number of complex molecules (11 biopolymers), including Y-cyclodextrin, pectin, gelatine, laminarin and mannan. They showed potential for application in biotechnology involving biopolymer hydrolysis such as the manufacture of artificial antigens, chemical diagnostics agents, release of oligosaccharides and oligopeptides in (ultra)oligotrophic freshwater environments. They also find application in the food and stockfeed technology, coffee extraction, oil drilling and detergent industries.

6.5. Acknowledgements

We wish to acknowledge Dr. A. Chouankam, BIOLOG Inc. Technical Department, for his assistance with data analysis.

CHAPTER 7

DEGRADATION OF POULTRY FEATHER WASTE BY *Chryseobacterium carnipullorum* 9_R23581^T

Abstract

Feather waste is produced in large amounts as a by-product of poultry processing plants and causes serious disposal problems. Keratin makes up over 90% of feathers but it is not readily digestible by animals, and is resistant to proteases and insects. This is because keratin is made up of tightly packed α–helix and β–sheets which are assembled into supercoiled polypeptide chains that are extensively cross-linked by cystine bridges, hydrogen bonds and hydrophobic interactions. A very small percentage of feather waste is steamed, treated chemically and ground to form dietary protein supplement for animals. Biological degradation of feathers using keratinolytic organisms is an economical and environmentally friendly alternative. *Chryseobacterium carnipullorum* 9_R23581^T, previously isolated from raw chicken, almost completely degraded all feathers in a feather meal medium within 48 h. Proteolytic and keratinolytic activities were detected in culture supernatants using azocasein and azokeratin respectively. The maximum protease activity was 110 U/ml after 48 h and the maximum keratinolytic activity was 22 U/ml on day 3. A maximum protein content of 2.58 mg ml^{-1} on day 3 was achieved. Such a novel keratinolytic isolate has potential biotechnological use in processes involving keratin hydrolysis.

7.1. Introduction

Feathers make up five to seven percent of the total weight of mature chickens (Santos *et al*., 1996). They are produced in huge quantities as a waste by-product by poultry processing plants. In India alone, feathers are produced at a rate of 350 million tonnes per annum (Agrahari and Wadhwa, 2010). In Japan, they are produced at a rate of 50 000 tonnes each year (Kojima *et al*., 2006), while in the United States of America, they are produced at a rate of 22 000 tonnes annually (Joshi *et al*., 2007). They are difficult to degrade and therefore pose a mammoth disposal problem. The main feather waste disposal methods include burning or burial but the main disadvantage of these methods is that they are not environmentally friendly because they pollute the air, soil and water (Joshi *et al*., 2007). A small

proportion of feather waste is steamed, chemically treated and ground, to form feather meal which is used as a protein supplement for animals (Agrahari and Wadhwa, 2010).

Feathers are a potential alternative to the more expensive dietary ingredients used in stock feeds since they are cheap, readily available and are made up of at least 90% keratin. However, keratin is not easily digestible and is stable in the presence of the digestive enzymes trypsin and pepsin as well as other proteolytic enzymes such as papain. This is because keratin is made up of tightly packed α–helix or β–sheets which are assembled into supercoiled polypeptide chains that are extensively cross-linked by cystine bridges, hydrogen bonds and hydrophobic interactions (Santos *et al*., 1996; Parry and North, 1998).

Proteases are collectively known as peptidyl-peptide hydrolases and they are classified into acidic, neutral and alkaline proteases. The latter constitute one of the most important groups of enzymes (Nagal *et al*., 2010). They have a potential to be used in non-polluting biotechnological processes in the poultry and leather industries (Shih, 1993; Onifade *et al*., 1998). Alkaline keratinases can be used to convert feathers to feedstuffs, fertilizers and polymers. They can also be used in the production of rare amino acids such as serine, cystein and proline (Papadopoulos *et al*., 1986; Yamauchi *et al*., 1996).

Several researchers have reported on microorganisms that are capable of degrading feather keratin. Santos *et al*. (1996) showed that *Aspergillus fumigatus* Fresenius can utilize chicken feather keratin as its sole carbon and nitrogen source. However, this organism is an opportunistic airborne pathogen affecting humans, birds and other animals. Lee *et al*. (1991), Kim *et al*. (2001) and Korkmaz *et al*. (2004) reported keratinolytic isolates belonging to the genus *Bacillus*. A keratinolytic serine protease from *Streptomyces pactum* DSM40530 was characterized by Bockle *et al*. (1995). Feather-degrading Gram-negative strains of *Vibrio* sp., *Stenotrophomonas* sp., and *Chryseobacterium* kr6 were isolated from

chicken feather waste (Sangali and Brandelli, 2000; Yamamura *et al*., 2002; Riffel *et al*., 2003). Nagal and Jain (2010) isolated *Elizabethkingia meningoseptica* KB042 from dropped off feathers.

The genera *Chryseobacterium* and *Elizabethkingia* have been associated with proteolytic activity (Vandamme *et al*., 1994a). The aim of this study was to evaluate the potential application of the previously isolated and described *Chryseobacterium carnipullorum* sp. nov. which was isolated from raw chicken (Chapters 4 and 5) by determining its proteolytic and keratinolytic activities. Chicken feather medium was used as the growth substrate.

7.2. Materials and Methods

7.2.1. *Organisms used*

The test organism was *C. carnipullorum* 9_R23581^T. The following organisms were used as references: The closest phylogenetic neighbour for *C. carnipullorum* 9_R23581^T, *C. shigense* DSM 17126^T; the type species *C. gleum* NCTC 11432^T; and positive keratinolytic species *Streptomyces* sp. DSM 40758 and *B. cereus* ATCC 10876^{TM}. Several attempts to obtain *Chryseobacterium* sp. kr6 which was reported to be very keratinolytic (Riffel and Brandelli, 2006), were unsuccesfull.

7.2.2. *Preparation of whole-feather medium*

Whole feathers collected from a local poultry processing plant were washed using detergent (sumanol) and rinsed thoroughly with tap water. They were sun-dried for 24 h followed by drying in an oven at 65^{o}C for 72 h (Santos *et al*., 1996). One gram of the chicken feathers was suspended in 100 ml basal medium consisting of (g/l): K_2HPO_4, 0.4; $MgSO_4.7H_2O$, 0.05; NaCl, 0.05 and $FeCl_3$, 0.01; pH 7.5 in 500 ml Erlenmeyer flasks. The flasks with medium were sterilized at 121 ^{o}C, 15 lbs per square inch (psi) steam pressure for 15 min (Nagal *et al*., 2010).

7.2.3. *Enzyme production*

The production of enzyme was done according to Nagal *et al*. (2010) with some modifications. One millilitre of a 24 h bacterial culture grown in Nutrient Broth (Oxoid) at 25 ^{o}C was used to inoculate 100 ml of the whole-feather medium (1% v/v). One millilitre of sterilized 24 h *Streptomyces* sp. DSM 40758 and *B. cereus* ATCC 10876^{TM} cultures were added to the control flasks. The flasks were then incubated at 25 ^{o}C and 120 rpm for 5 days.

To study the effect of incubation period on the course of alkaline protease production during the growth of the organisms in whole feather medium, 2 ml samples were withdrawn at 24 h intervals up to 5 days. In each case, a clear supernatant was obtained by filtering through a Whatman filter paper no. 1. Samples were centrifuged at 10 000 x *g* for 10 min at 4 ^{o}C to remove cells and insoluble feather fragments. The supernatant was used as the crude enzyme preparation. All experiments were carried out in triplicate and results were reported as mean values (Nagal and Jain, 2010).

7.2.4. *Measurement of enzyme activity*

7.2.4.1. *Proteolysis*

The proteolytic activity was assayed by the azocasein method according to Christen and Marshal (1984) and Deeth *et al*. (2002) with some modifications. The cell free extract (250 µl) was added to 1 ml of 1% azocasein (Sigma-Aldrich A2765; 10 g/l dissolved in phosphate buffer pH 7.2). For the control sample, 250 µl of phosphate buffer pH 7.2 was added instead of the enzyme. The reaction mixture was incubated for 1 h at 37 ^{o}C and the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 100 g/l. After centrifugation at 10 000 x *g* for 15 min, the absorbance of the supernatant was measured at 345 nm.

7.2.4.2. *Keratinolysis*

The keratinolytic activity was determined according to Wang *et al*. (2008) with some modifications. Keratin azure (Sigma-Aldrich, K8500) was used as the insoluble substrate (Riffel *et al*., 2007). The reaction mixture contained 500 µl of enzyme preparation and 500 µl of 50 mM sodium phosphate buffer (pH 8) containing 5 mg of keratin azure. The mixture was incubated at 37 ^{o}C for 30 min and the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 100 g/l. After centrifugation at 10 000 x *g* for 5 min, the absorbance of the supernatant was measured at 440 nm (Sangali and Brandelli, 2000; Riffel *et al*., 2003). Control samples were prepared in a similar manner except that the enzyme was replaced by the sodium phosphate buffer. The assays were conducted in triplicate. One unit of enzyme was defined as the amount of enzyme that resulted in an increase in absorbance at 440 nm (A_{440}) of 0.01 after reaction at 37 ^{o}C for 30 min with keratin azure.

7.2.5. *Determination of protein content of the bacterial culture filtrates*

Protein determination was done in duplicate according to the Biuret method (Layne, 1957) with bovine serum albumin, fraction V (Sigma) as the standard.

7.3. Results and discussion

Feather degradation was confirmed visually. It was observed that aerobic growth of *C. carnipullorum* 9_R23581^{T} in the whole feather medium, with the feathers as its primary source of carbon, nitrogen, energy and sulphur, resulted in almost complete degradation of feather keratin after incubation for 48 h at 25 ^{o}C compared to the reference strains (Figure 7.1). Feather barbules were completely hydrolyzed and rachises were extensively degraded. This observation was similar to reports by other researchers who showed that *Chryseobacterium* strains such as *C. indologenes* 1x9a (Venter *et al*., 1999), *Chryseobacterium* sp. (Lijnen *et al*., 2000) and *Chryseobacterium* sp. kr6 (Brandelli and Riffel, 2005) as well as

Elizabethkingia meningoseptica KB042 (Nagal *et al*., 2010), produced alkaline proteases at mesophilic temperatures. The enzymes produced by the mentioned organisms showed keratinolytic activity.

The reference strains [*C. shigense* DSM 17126^{T} and *C. gleum* NCTC 11432^{T}, 72 h; *Streptomyces* sp. DSM 40758 and *B. cereus* ATCC 10876^{TM}, 96 h (Figure 7.2)] exhibited the same visual feather degradation as seen with *C. carnipullorum* 9_R23581^{T} after 48 h. The positive keratinolytic reference strains, *Streptomyces* and *B. cereus*, were selected because of their well documented keratinolytic activities as well as their availability (Williams *et al*., 1990; Bockle *et al*., 1995; Onifade *et al*., 1998, Kim *et al*., 2001; Matikeviciene *et al*., 2009, Agrahari and Wadhwa, 2010).

A time course study of protease activity at 25 ^{o}C produced by *C. carnipullorum* 9_R23581^{T}, *C. shigense* DSM 17126^{T}, *C. gleum* NCTC 11432^{T}, *Streptomyces* sp. DSM 40758 and *B. cereus* ATCC 10876^{TM}, is shown in Figure 7.3. The highest proteolytic activity (188 U/ml) was obtained with *C. gleum* at 24 h of incubation. *C. shigense* gave the second highest activity (111 U/mg) at 24 h.

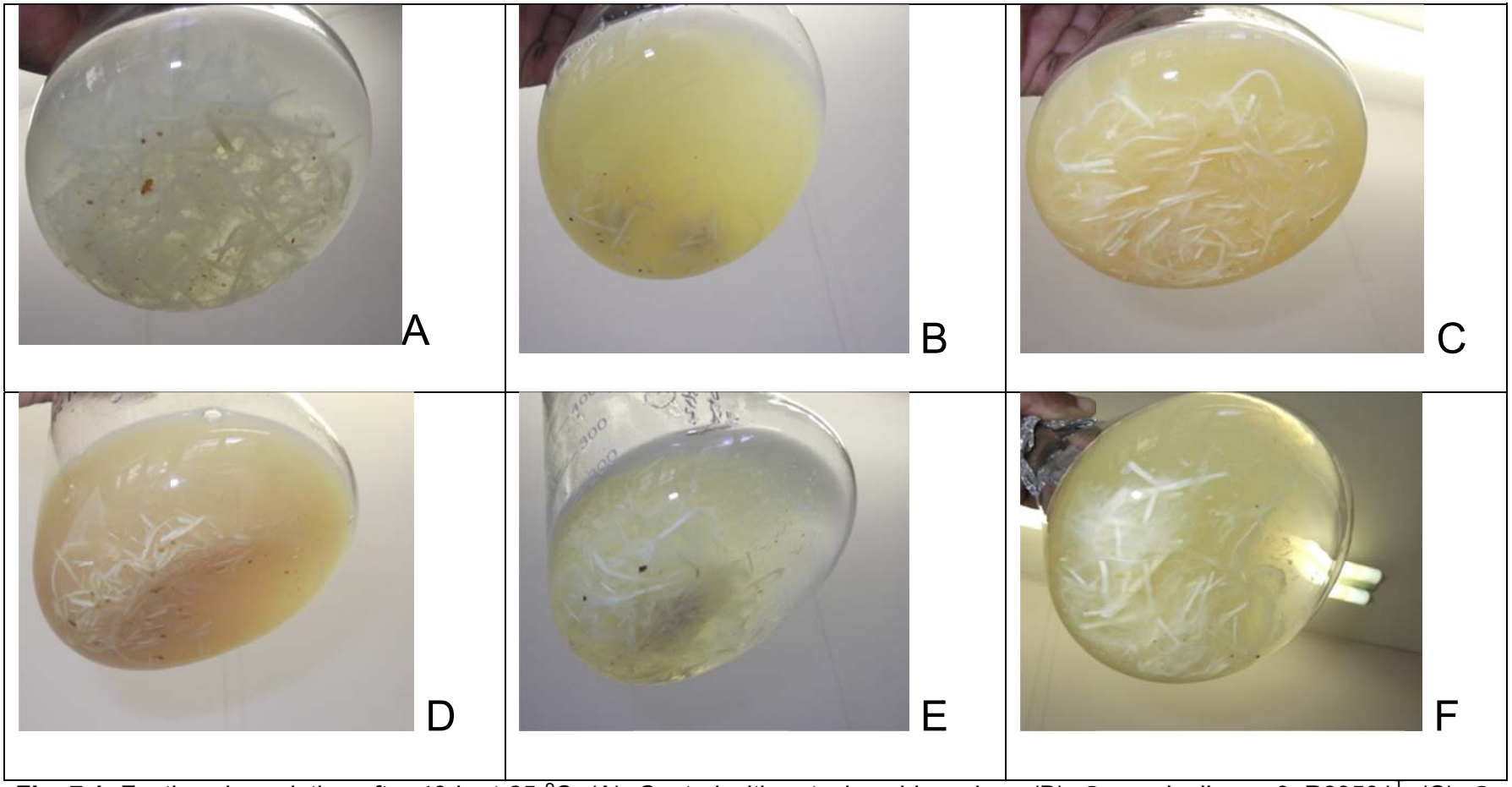


Fig. 7.1. Feather degradation after 48 h at 25 ^{o}C: (A), Control with autoclaved inoculum; (B), *C. carnipullorum* 9_R23581^{T}; (C), *C. shigense* DSM 17126^{T}; (D), *C. gleum* NCTC 11432^{T}; (E), *Streptomyces* sp. DSM 40758; (F), *B. cereus* ATCC 10876^{TM}.

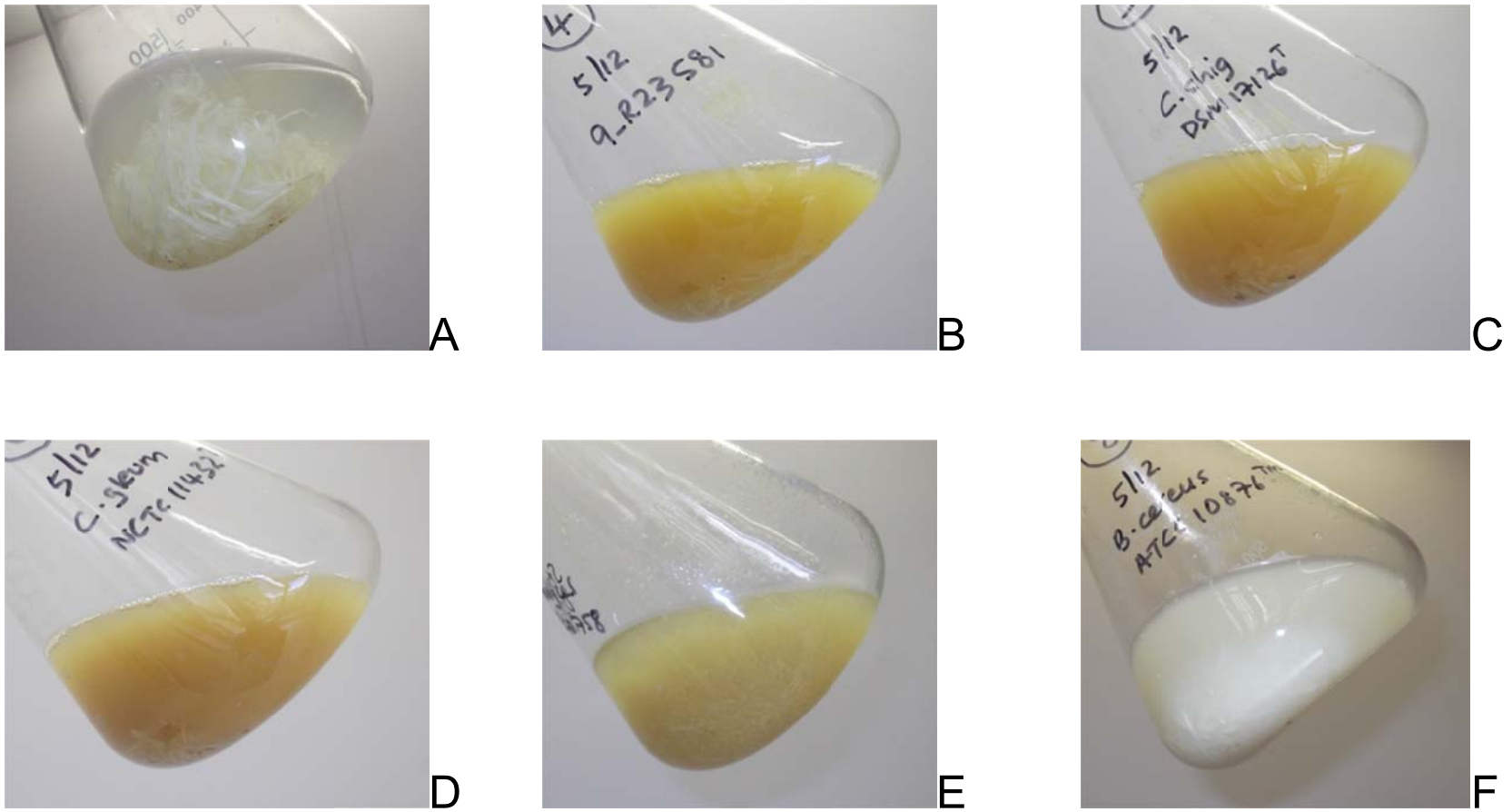


Fig. 7.2. Optimum feather degradation at 25 ^{o}C: (A), Control with autoclaved inoculum; (B), *C. carnipullorum* 9_R23581^{T} day 2; (C), *C. shigense* DSM 17126^{T} day 3; (D), *C. gleum* NCTC 11432^{T} day 4; (E), *Streptomyces* sp. DSM 40758 day 5; (F), *B. cereus* ATCC 10876^{TM} day 5.

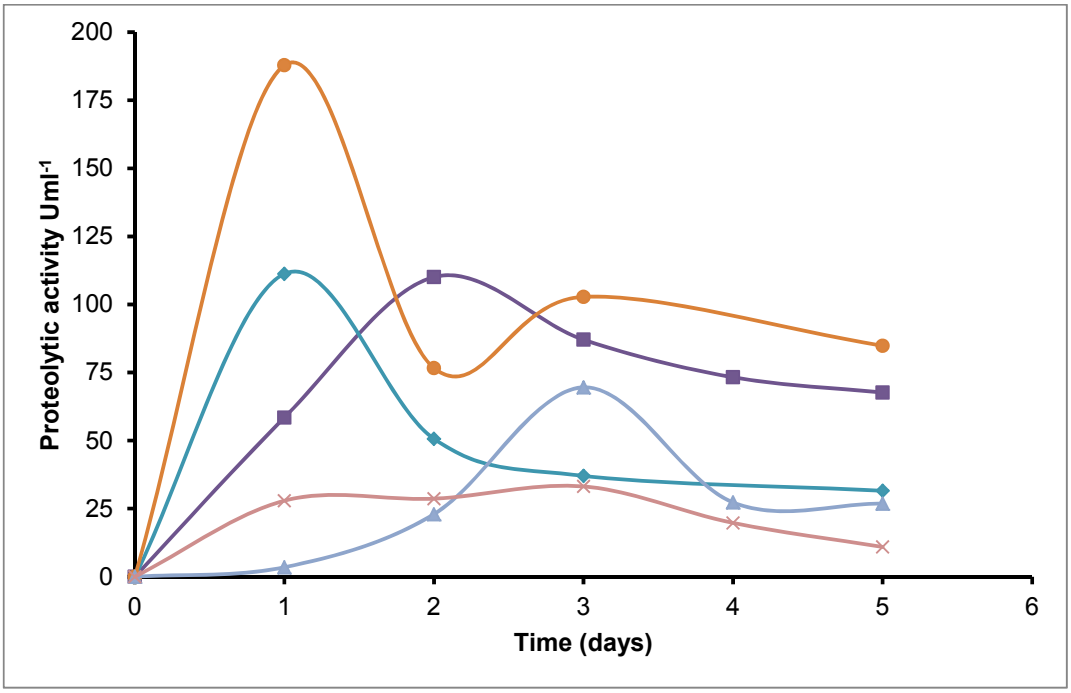


Fig. 7.3. Proteolytic activity during growth of bacteria in feather broth medium at 25 °C. Enzyme activities were measured using azocasein as the substrate. Each point represents the mean of three determinations. (■) *C. carnipullorum* 9_R23581^T; (♦) *C. shigense* DSM 17126^T; (●) *C. gleum* NCTC 11432^T; (▲) *Streptomyces* sp. DSM 40758; (×) *B. cereus* ATCC 10876^{TM}.

Protease activity by *C. carnipullorum* 9_R23581^T was third highest and similar to that for *C. shigense* DSM 17126^T peaking at 110 U/ml after 48 h. The activities for *Streptomyces* sp. DSM 40758 (70 U/ml) and *B. cereus* ATCC 10876^{TM} (33 U/ml) were the fourth and fifth best, respectively.

The proteolytic activity of enzymes produced by *C. carnipullorum* 9_R23581^T was consistent with the majority of *Chryseobacterium* strains which were reported to produce highly proteolytic enzymes (Jooste *et al*., 1986; Yamaguchi and Yokoe, 2000).

A time course study of keratinolytic activity (Figure 7.4) revealed that *C. carnipullorum* 9_R23581^T gave the highest keratinase activity of 22 U/ml on day 3.

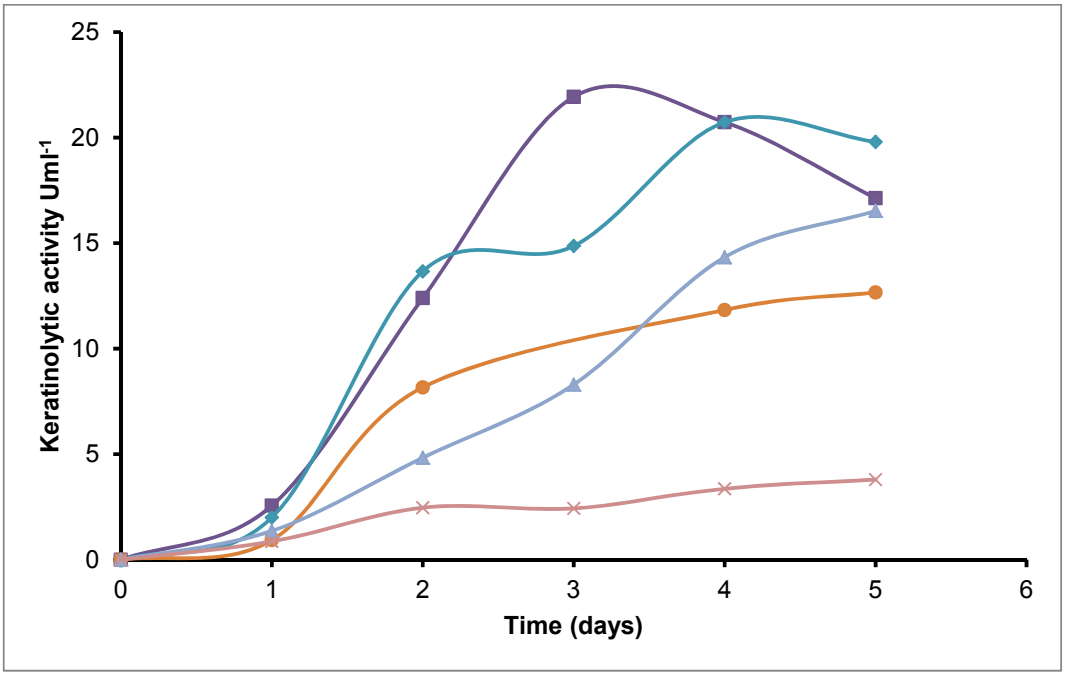


Fig. 7.4. Keratinolytic activity during growth of bacteria in feather broth medium at 25 °C. Enzyme activities were measured using azokeratin as the substrate. Each point represents the mean of three determinations. (■) *C. carnipullorum* 9_R23581^T; (♦) *C. shigense* DSM 17126^T; (●) *C. gleum* NCTC 11432^T; (▲) *Streptomyces* sp. DSM 40758; (×) *B. cereus* ATCC 10876^{TM}.

This was followed by the activity of keratinases produced by *C. shigense* DSM 17126^T of 21 U/ml at day 4. Keratinases produced by *Streptomyces* sp. DSM 40758 gave a peak activity of 17 U/ml at day 5. The keratinolytic activities for *C. gleum* NCTC 11432^T (13 U/ml) and *B. cereus* ATCC 10876^{TM} (4 U/ml) were the fourth and fifth best, respectively. The maximum keratinolytic activity of *C. carnipullorum* 9_R23581^T at 25 °C was lower but comparable to that reported for *Chryseobacterium* kr6 (approximately 28 U/ml) at the same assay temperature (Riffel *et al*., 2003). However, *C. carnipullorum*9_R23581^T gave a much higher activity at day 3 compared to *C. indologenes* TKU014 (10 U/ml) at the same feather concentration (Wang *et al*., 2008). The differences in these results may be due to the differences in the methods used as well as the strains concerned. Substrate

concentration also plays a critical role since keratinases are substrate specific and inducible, and high substrate concentrations may cause substrate inhibition or repression of keratinase production (Brandelli and Riffel, 2005; Wang *et al*., 2008). Therefore, there is a need to investigate optimum conditions for production of the keratinase, such as substrate concentration, temperature and pH, purification and characterization of the keratinase, even though these aspects are not part of the present study.

Protein content of the filtrates of the cultured strains is shown in Table 6.1. The protein content for *C. carnipullorum* 9_R23581^{T} ranged from 1.72 to 2.58 mg ml^{-1}. *Chryseobacterium shigense* DSM 17126^{T} had protein content ranging from 2.00 to 2.57 mg ml^{-1}, with the maximum being attained on day 4. The protein content for *C. gleum* NCTC 11432^{T} ranged from 1.88 to 2.10 mg ml^{-1} while *Streptomyces* sp. DSM 40758 and *B. cereus* ATCC 10876^{TM} ranged from 1.97 to 2.89 mg ml^{-1} and 1.60 to 2.06 mg ml^{-1} respectively.

Even though *C. carnipullorum* 9_R23581^{T} gave a maximum protein content (2.58 mg ml^{-1}, day 3) second only to *Streptomyces* sp. DSM 40758, it had a time advantage over the slow-growing *Streptomyces* sp. DSM 40758 which reached a maximum protein value (2.89 mg ml^{-1}) only on day 5. Protein contents of the culture filtrates produced by *Elizabethkingia meningoseptica* KB042 (1.432 mg ml^{-1}; Nagal *et al*., 2010), *B. licheniformis* 511 (1.14 mg ml^{-1}) and *B. subtilis* I-1 (1.14 mg ml^{-1}; Matikeviciene *et al*., 2009), were lower than that for *C. carnipullorum* 9_R23581^{T}.

Microbial keratinases improve the digestibility of feather keratin (Lee *et al*., 1991). The ability of *C. carnipullorum* 9_R23581^{T} to produce keratinolytic activity that can attack the compact feather structure releasing proteins can be exploited during the manufacture of animal feed. Soy meal, fish meal, blood meal and meat and poultry meal are some of the common protein sources used in the production of animal feed. However, these are generally expensive compared to the abundant feather waste. Moreover, feather waste has the highest amount of protein, but animals cannot readily digest it. It is not used extensively in animal feed production since current

treatments destroy the heat-sensitive amino acids thereby lowering the quality of the animal feed (Riffel and Brandelli, 2006; Casarin *et al*., 2008).

Use of *C. carnipullorum* 9_R23581^T as a keratin degrader may alleviate the feather waste disposal problem currently being experienced by the poultry processing industry. The organism's keratinases also have huge potential in non-polluting biotechnological processes involving keratin hydrolysis. After hydrolysis, the feathers can be converted to fertilizers, glues, films, and as the source of rare amino acids, such as serine, cysteine and proline (Gupta and Ramnani, 2006).

Table 7.1. The protein content of filtrates of *C. carnipullorum* 9_R23581^T, *C. shigense* DSM 17126^T, *C. gleum* NCTC 11432^T, *Streptomyces* sp. DSM 40758 and *B. cereus* ATCC 10876^{TM}. cultured in feather meal medium. Each value is the mean of two determinations.

	Protein content (mg ml^{-1})				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
C. carnipullorum 9_R23581^T	1.72	2.02	2.58	2.40	2.31
C. shigense DSM 17126^T	2.00	2.18	2.43	2.57	2.54
C. gleum NCTC 11432^T	1.88	2.00	2.04	2.00	2.10
Streptomyces sp. DSM 40758	1.97	2.05	2.50	2.86	2.89
B. cereus ATCC 10876^{TM}	1.60	1.88	1.96	2.06	1.99

7.4. Conclusions

The results of the present study show that the crude enzyme produced by *C. carnipullorum* 9_R23581^T was both proteolytic and keratinolytic. Keratinolysis was confirmed visually and feather degradation could be seen. The highest proteolytic activity against azocasein (110 U/ml) was obtained after 48 h while the highest keratinolytic activity against azokeratin (22 U/ml) was reached after 72 h. Keratinases have great potential biotechnological

applications in industries such as poultry processing, stock feed production and leather manufacturing. Future work should include optimisation of enzyme production, purification and characterization of the keratinase of *C. carnipullorum* 9_R23581^{T}, analysis of free amino acids and effect of inhibitors.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1. Introduction

Species belonging to the genus *Chryseobacterium* are widely distributed in clinical (Shewan and McMeekin, 1983; Yabuuchi *et al*., 1983; Holmes *et al*., 1984b) and environmental sources. In environmental sources, they are found in water, soil and food ecosystems (Vandamme *et al.,* 1994b; Jooste and Hugo, 1999; Bernardet *et al.,* 2006). Environmental strains have been isolated from fresh water and lake sediments (Kim *et al*., 2005b; 2008); drinking water (Gallego *et al*., 2006); soil (Yamaguchi and Yokoe, 2000); polar regions (Yi *et al*., 2005) and rhizosphere of plants (Park *et al*., 2006). Industrial strains have been isolated from sources such as activated sewage sludge (Kämpfer *et al*., 2003). *Chryseobacterium* sp. are also commonly encountered in food environments. These sources include the dairy environment, such as fresh cow milk in South Africa and Israel (Jooste, 1985; Jooste *et al*., 1986; Hugo *et al*., 2003; Hantsis-Zacharov *et al*., 2008a; 2008b) and a lactic acid beverage in Japan (Shimomura *et al*., 2005). They have also been isolated from meat and poultry products (García-López *et al*., 1998; de Beer *et al*., 2005). Feathers, skins and intestines are the main sources of contamination of poultry by these organisms (Mead, 1989).

The genus *Chryseobacterium* belongs to the family *Flavobacteriaceae* (Bernardet *et al*., 2006). The genus name was proposed by Vandamme *et al.* (1994a) based on rRNA studies. At that juncture, it consisted of six species (*Chryseobacterium balustinum*, *C. gleum* [the type species], *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum*) that were relocated from the erstwhile *Flavobacterium* genus. This genus belongs to the smaller of the two well-defined clades in the family comprising non-gliding, non-halophilic, and mostly non-psychrophilic organisms as well as most unpigmented and several pathogenic members of the family (see Fig. 2.1 in Chapter 2). The other genera in this clade

are *Elizabethkingia*, *Riemerella*, *Bergeyella*, *Kaistella*, *Sejongia*, *Epilithonimonas*, *Ornithobacterium*, *Empedobacter* and *Weeksella* as well as a variety of poorly characterized organisms (Bernardet *et al*., 2011). The suggestion of the family *Flavobacteriaceae* by Jooste (1985) introduced much homogeneity to the taxonomy of the flavobacteria. Currently there are 94 genera (Euzéby, 2012b), of which only 10 are associated with food (Hugo and Jooste, 2012).

Even though the significance of the genus *Chryseobacterium* in the food industry has long been debated, it was found that the organisms can cause post-sterilization contamination in vegetable canning plants and a variety of defects in dairy products such as surface taint and apple odour in butter (Jooste *et al*., 1986; Hugo, 1998). The heat stable metalloproteases produced by these organisms may also cause spoilage problems in the dairy industry (Venter *et al*., 1999). Recently, it was found that some strains of *Chryseobacterium* were also able to break down indigestible and virtually non-biodegradable feather keratin, releasing proteins and amino acids which could easily be digested as nutritious feather meal stockfeed supplements (Riffel *et al.,* 2003; Casarin *et al.,* 2008).

The aims of this study were, firstly, to isolate yellow-pigmented isolates from chicken feather waste and feather meal samples from poultry processing plants and to screen for *Chryseobacterium* species with a view to finding species/strains that were capable of degrading feather waste. Secondly, the study sought to classify the *Chryseobacterium* strains isolated from poultry feather waste and raw chicken (Chapter 3). The techniques that were employed in the latter exercise were traditional phenotypic identification and characterization techniques using the BIOLOG Gen II microbial identification system but also 16S rRNA gene sequence analysis. Thirdly, a polyphasic taxonomic approach (Vandamme *et al*., 1996) was followed to determine whether any of the unidentified strains belonged to new species. Techniques included in this polyphasic approach included the determination of DNA base composition by HPLC, spectroscopic DNA-DNA hybridization, fatty acid and quinone analysis by TLC and advanced phenotypic characterization using the BIOLOG Gen III microbial identification system. A new species which did emerge namely, *Chryseobacterium carnipullorum* sp. nov., was described. This novel species was then subjected to advanced “global”

phenotypic characterization, to pursue a fourth aim using the latest technology developed by BIOLOG Inc., namely the Phenotype MicroArray system, to shed light on the possible spoilage characteristics and application potential of this organism. The fifth and final aim was to investigate the application potential of the new species to the degradation of feather waste keratin.

8.2. Isolation of *Chryseobacterium* species from poultry feather waste

Samples for the envisaged isolation of the genus *Chryseobacterium* were collected from two poultry processing plants in Bloemfontein (A and B), the Free State province, and the third one (C) in Gauteng province, South Africa. This genus typically consists of Gram-negative, oxidase positive, non-endospore-forming, non-motile and yellow-pigmented rods (Bernardet *et al*., 2011). The methods used to screen for *Chryseobacterium* species were according to MacFaddin (1980) as shown in Table 3.2. Of the different incubation conditions used during the isolation of the yellow-pigmented strains (4 ^{o}C for 10 days; 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h and 25 ^{o}C for 48 h), the highest total yellow colony counts (TYCC) were obtained after incubation at 4 ^{o}C for 48 h followed by 25 ^{o}C for 48 h. This incubation regime was advantageous to the selection of the yellow-pigmented chryseobacterial species which would otherwise have been out-grown if a higher incubation temperature had been used from the beginning.

Even though the TYCC on Standard Plate Count Agar was higher (mean of 6 log cfu g^{-1}) than the counts on Nutrient Agar (mean of 5 log cfu g^{-1}), they were comparable. The buried feather samples revealed that the yellow-pigmented (aerobic) species did not survive well after being buried for more than one week. Overall, a TYCC of 10.5 log cfu g^{-1} was obtained and representive sampling of colonies from these plates yielded 466 yellow isolates. After screening, presumptive *Chryseobacterium* estimated at 6.1 log cfu g^{-1} and representive sampling yielded 35 isolates. Only seven of these isolates could be satisfactorily identified by the BIOLOG Gen II identification system. This, however, was attributable to the small *Chryseobacterium* data base in the BIOLOG system and the fact that the Microlog software reported new and atypical species as “No ID” (BIOLOG Inc., 2001). Twelve unidentified isolates were selected for subsequent

studies together with 17 unidentified presumptive *Chryseobacterium* isolates from a previous study (de Beer, 2005), providing a total of 29 isolates.

8.3. Classification of *Chryseobacterium* strains isolated from poultry feather waste and raw chicken

The techniques that were employed in this part of the study were 16S rRNA gene sequencing and the BIOLOG Omnilog Gen III identification system. The 16S rRNA gene sequencing technique is a very powerful and universal molecular tool for classification of isolates. It provides the relationship between unknown strains and the reference strains and delineates up to genus level (Drancourt *et al*., 2000). The BIOLOG Omnilog Gen III identification system was used for identification as well as phenotypic characterization of the isolates.

8.3.1. *16S rRNA gene sequence analysis*

16S rRNA gene sequencing was able to identify 14 of the 29 isolates tested as belonging to the genus *Chryseobacterium.* Seven of the 14 identified isolates which had ≤97% 16S rRNA gene sequence similarity were sequenced using two more internal primers. It became evident after the phylogentic tree was constructed that the seven isolates, 1_F178, 5_R23647, 6_F141B, 7_F195, 8_R23573, 9_R23581 and 10_R23577, were indeed closely affiliated to the genus *Chryseobacterium*.

Isolate 1_F178, which was obtained from feather waste that had been land-filled for 1 week (Chapter 3), had *C. jejuense* as its nearest phylogenetic neighbour. This phylogeny was moderately supported by a bootstrap re-sampling value of 68%. The closest phylogenetic neighbours for isolate 5_R23647 were *C. piscium*, *C. balustinum* and *C. scophthalmum*. This phylogeny's stability was supported by a bootstrap re-sampling value of 81%. Isolate 5_R23647 was a raw chicken isolate which clustered with species that were isolated from diseased and/or healthy fish (Mudarris *et al*., 1994; de Beer *et al*., 2005; Bernardet and Nakagawa, 2006). Isolates 6_F141B and 7_F195 formed a tight cluster. This suggested that the strains could belong to the same species. They had *C. flavum*

as their nearest phylogenetic neighbour. This phylogeny was supported by a very high bootstrap re-sampling value of 100%. Isolates 8_R23573, 9_R23581 and 10_R23577 formed a tight cluster that was supported by a bootstrap re-sampling value of 85%. This suggested that the strains could belong to the same species. Their nearest phylogenetic neighbour was *C. shigense* GUM Kaj^T. The above phylogenies were also obtained using alternative treeing methods (the maximum parsimony algorithm and UPGMA analysis in the MEGA version 4 software package; Tamura *et al*., 2007) although at different bootstrap support values.

8.3.2. *Phenotypic classification*

Both conventional phenotypic tests and the BIOLOG Omnilog Gen III identification system were employed. The nearest phylogenetic neighbours were used as reference organisms and if they were not available, *C. gleum* (the type species) was used as recommended by Tindall *et al*. (2010).

It became clear that the seven unidentified isolates could be regarded as four different species represented by the following strains: 1_F178; 5_R23647; 6_F141B and 7_F195; and 8_R23573, 9_R23581 and 10_R23577. These phenotypic observations supported earlier findings from phylogenetic analysis. The BIOLOG Omnilog Gen III identification system is a reliable technology since it was able to identify all reference strains that are in its data base. Its major limitation was the small database for the genus *Chryseobacterium* which currently has five species compared to 58 species that are known to date (Euzéby, 2012a).

8.4. Polyphasic taxonomic study of raw chicken isolates and description of *C. carnipullorum* sp. nov.

The isolates, 8_R23573, 9_R23581 and 10_R23577, were evaluated using the following techniques: the determination of DNA base composition by HPLC, spectroscopic DNA-DNA hybridization, fatty acid and quinone analysis by TLC. The predominant respiratory quinone was menaquinone MK-6 which was 97, 97 and 98% respectively. This was consistent with this characteristic in the genus *Chryseobacterium* (Bernardet *et al*., 2011). The predominant cellular fatty acids

were 15:0 iso, iso 17:1 ω9c, 17:0 iso 3OH and summed feature 3, consisting of 16:1 ω7c/15 iso 2OH, also supported the affiliation of the strains to the genus *Chryseobacterium.* DNA base composition for the genus *Chryseobacterium* ranges from 29 to 39 mol % G+C (Bernardet *et al*., 2011). Therefore, the DNA base composition of the isolates (36.9, 36.7 and 36.6 mol % G+C respectively) further supported the affiliation of the strains to the genus *Chryseobacterium*.

It became evident that a new species had emerged when the DNA-DNA hybridization results were considered. The strains had relatedness values of more than 81% among the three strains and less than 57% similarity between the strains and the two nearest phylogenetic neighbours *Chryseobacterium shigense* (DSM 17126^T) and *Chryseobacterium luteum* (LMG23785^T; Table 5.2). This provided evidence for the description of a novel species when recommendations of a threshold value of 70 % DNA-DNA similarity for the delineation of bacterial species were considered (Wayne *et al*., 1987) and keeping in mind all previous phenotypic findings. The name *Chryseobacterium carnipullorum* sp. nov. was proposed for the new species. It consists of three strains, 8_R23573, 9_R23581 and 10_R23577, of which strain 9_R23581 was chosen as the type strain.

8.5. Phenotype MicroArray characterization of *Chryseobacterium carnipullorum* R23581^T

The BIOLOG Omnilog Phenotype MicroArray technology was used to globally phenotype *C. carnipullorum* 9_R23581^T with *C. shigense* DSM 17126^T and *C. gleum* NCTC 11432^T as reference organisms. This technology tests 1920 different phenotypes at the same time. *Chryseobacterium carnipullorum* 9_R23581^T and *C. shigense* DSM 17126^T differed by 1.9% (37/1 920) while *C. carnipullorum* 9_R23581^T and *C. gleum* NCTC 11432^T differed by 1.2% (23/1 920). The differences occurred in the following substrate classes: antifungals, alcohols, amides, amino acids, carbohydrates, polymers, a lipophilic chelator, esters, nitrite, cephalosporins and antibiotics.

In this study, substrate utilization gave an indication of broader potential applications of the novel species *C. carnipullorum* 9_R23581^T. The substrates

mostly utilized were carbohydrates (67) followed by carboxylic acids (59) and amino acids (30). This implies that the organism has a high potential to cause food spoilage emanating from metabolites of the food nutrients. Carbohydrate metabolites such as organic acids, alcohols and carbon dioxide may cause souring and butyric spoilage defects, softening and liquefaction can be be caused by random splitting of glycosidic bonds. Incomplete oxidation of carboxylic acids may cause sour tastes while amino acid metabolites may cause putrescence and sulphide spoilage (Ayers *et al*., 1980; Coultate, 1984; Frazier, 1988; Banwart, 1989).

Chryseobacterium carnipullorum 9_R23581^T can produce enzymes which are capable of degrading complex molecules. Polymers such as Y-cyclodextrin can be hydrolysed to maltooligomers which find application in the preparation of artificial antigens (Jodal *et al*., 2011). Other polymers occurring in fresh water such as amylopectin, gelatine and laminarin can be degraded to oligosaccharides and oligopeptides thereby releasing nutrients that can be utilized by other species in (ultra) oligotrophic freshwater environments. Mannases produced by *C. carnipullorum* 9_R23581^T may find applications in food and stockfeed technology, coffee extraction, oil drilling and detergent industries (Dhawan *et al*., 2008).

8.6 Degradation of poultry feather waste by *Chryseobacterium carnipullorum* R23581^T

The genus *Chryseobacterium* has long been associated with proteolytic activity (Jooste *et al*., 1986; Venter *et al*., 1999; Lijnen *et al*., 2000). In this project, it was established that *C. carnipullorum* R23581^T produced extracellular proteases which were both proteolytic and keratinolytic. The organism was able to visibly almost completely degrade feathers in 48 h. This was faster than the degradation rates reported for other documented keratinolytic organisms such as *Chryseobacterium* sp. kr6 (72 h; Riffel *et al*., 2003) and *Bacillus licheniformis* PWD1 (10 days; Williams *et al*., 1990). The maximum protease activity was 110 U/ml after 48 h and the maximum keratinolytic activity was 22 U/ml on day 3.

Degradation of feather waste is critical because feathers are produced in huge quantities annually. They are not easily degradable by common proteases such as trypsin and this causes serious disposal problems. Hydrolysed feathers can also provide proteins and amino acids for feedstocks since feathers are made up of more than 90% keratin. The novel keratinolytic isolate has potential biotechnological applications in environmentally friendly processes involving keratin hydrolysis.

8.7 Conclusions and recommendations for future research

8.7.1. *Conclusions*

The following general conclusions could be drawn from this study:

i. *Chryseobacterium* species were found to occur on chicken feathers. A total of 466 yellow pigmented bacterial strains were isolated and classified using conventional methods and the BIOLOG Omnilog Gen II and Gen III identification systems. The conventional methods identified 35 of the isolates as presumptive *Chryseobacterium* species, but the number was reduced to 12 after BIOLOG Gen II and Gen III evaluations.

ii. Phenotypic and molecular techniques revealed the taxonomic affiliations of the isolates. Fourteen of the 29 isolates (12 isolated in this study and 17 isolated during a previous study; de Beer, 2005) were identified as belonging to the genus *Chryseobacterium* by 16S rRNA gene sequencing. A polyphasic taxonomic study confirmed the emergence of a new species, *C. carnipullorum* sp. nov., which was duly described and proposed.

iii. From the PM analysis, it became evident that *C. carnipullorum* 9_R23581^T can cause food spoilage since it mostly utilized commonly found nutrients such as carbohydrates, carboxylic acids and amino acids whose metabolites can lead to, inter alia, souring, butyric spoilage defects, alkalinization, bitter tastes and sulphide spoilage.

iv. The PM analysis also revealed that since *C. carnipullorum* 9_R23581^T showed its ability to degrade complex biopolymers, it has the potential for application in biotechnology involving biopolymer hydrolysis such as the manufacture of artificial antigens, chemical diagnostic agents, release of

oligosaccharides and oligopeptides in (ultra) oligotrophic freshwater environments. It also has potential for application in food and stockfeed technology, coffee extraction, oil drilling and detergent industries.

v. *Chryseobacterium carnipullorum* 9_R23581^{T}, isolated from a raw chicken portion, produced extracellular keratinases and it almost completely degraded feathers in a feather meal medium in 48 h. Therefore the organism has potential biotechnological applications in environmentally friendly processes involving keratin hydrolysis and effective disposal of feather waste.

8.7.2. *Recommendations for future research*

i. The isolates 1_F178; 5_R23647; 6_F141B and 7_F195 should be further investigated using the polyphasic approach to gather more information for description of possible new species.
ii. The above-mentioned isolates should be subjected to PM analysis to determine possible applications and potential spoilage characteristics.
iii. More work on the keratinolytic activity of the novel organism should include optimisation of enzyme production, purification and characterization of the keratinase, analysis of free amino acids and effect of inhibitors.

CHAPTER 9

REFERENCES

AFMA (Animal Feed Manufacturers Association). (2008). http://www.agri24.com/AFMA_Template/1,2491,6552_1646,00.html (Accessed on 18 November 2008).

Agrahari, S. & Wadhwa, N. (2010). Degradation of chicken feathers, a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. *Int J Poultry Sci* **9**(5), 482–489.

Alain, K., Intertaglia, L., Catala, P. & Lebaron, P. (2008). *Eudoraea adriatica* gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae*. *Int J Syst Evol Microbiol* **58**, 2275–2281.

Amann, R. I., Ludwig, W. & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59,** 143–169.

Arun, A. B., Chen, W. M., Lai, W. A., Chou, J. H., Shen, F. T., Rekha, P. D. & Young, C. C. (2009). *Lutaonella thermophila* gen. nov., sp. nov., a moderately thermophilic member of the family *Flavobacteriaceae* isolated from a coastal hot spring. *Int J Syst Evol Microbiol* **59**, 2069–2073.

Atalo, K. & Gashe, B. A. (1993). Protease production by a thermophilic *Bacillus* species (P-001A) which degrades various kinds of fibrous proteins. *Biotechnol Lett* **15**, 1151–1156.

Ayres, J. C., Mundt, J. O., and Sandine, W. E. (1980). *Microbiology of Foods*. W. H. Freeman and Company, San Fransisco.

Austin, B. & Priest, F. (1986). *Modern Bacterial Taxonomy*. Berkshire, U.K.: van Nostrand Reinhold.

Banwart, G. J. (1989). *Basic Food Microbiology*. London: Chapman & Hall.

Barbeyron, T., L'Haridon, S., Corre, E., Kloareg, B. & Potin, P. (2001). *Zobellia galactanovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and reclassification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 985–997.

Barrow, G. I. & Feltham, R. K. A. (1993). Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd edn. Cambridge: Cambridge University Press.

Behrendt, U., Ulrich, A., Sproer, C. & Schumann, P. (2007). *Chryseobacterium luteum* sp. nov., associated with the phyllosphere of grasses. *Int J Syst Evol Microbiol* **57**, 1881–1885.

Behrendt, U., Ulrich, A., Sproer, C. & Schumann, P. (2008). *Chryseobacterium gregarium* sp. nov., isolated from decaying plant material. *Int J Syst Evol Microbiol* **58**, 1069–1074.

Belak, A., Kovacs, M., Herman, Zs., Holczman, A. N., Marta, D., Stojakovic, S. C., Bajcsi, N. & Maraz, A. (2011). Molecular analysis of poutry meat spoiling microbiota and heterogeneity of their proteolytic and lipolytic enzyme activities. *Acta Alimentaria* **40**(1), 3–22.

Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W. & Huntoon, F. M. (1923). Genus II. *Flavobacterium* gen. nov. In *Bergey's Manual of Determinative Bacteriology*, 1st edn. pp. 97. Baltimore, MD: Williams & Wilkins.

Bergey, D. H., Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1939). Genus VI. *Flavobacterium* Bergey *et al.*, In *Bergey's Manual of Determinative Bacteriology*, 5th edn. pp. 522–553. London: Bailliere, Tindall & Cox.

Bergey, D. H. & Breed, R. S. (1948). Genus III. *Flavobacterium*. In *Bergey's Manual of Determinative Bacteriology*, 6th edn. pp. 427–430. Edited by R. S. Breed, E. G. D. Murray & A. P. Hitchens. Baltimore, MD: Williams & Wilkins.

Bernardet, J.-F., Kerouault, B. & Michel, C. (1994). Comparative study on *Flexibacter maritimus* strains isolated from farmed sea bass (*Dicentrarchus labrax*) in France. *Fish Pathol* **29**, 105–111.

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

Bernardet, J.-F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52,** 1049–1070.

Bernardet, J.-F., Vancanneyt, M., Matte-Tailliez, O., Grizez, L., Tailliez, P., Bizet, C., Nowakowski, M., Kerouault, B. & Swings, J. (2005). Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals. *Syst Appl Microbiol* **28**, 640–660.

Bernardet, J.-F., Hugo, C. J. & Bruun, B. (2006). The genera *Chryseobacterium* and *Elizabethkingia*. In *The Prokaryotes*, 7th edn, Ch. 6.12, pp. 638–676. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.

Bernardet, J.-F. & Nakagawa, Y. (2006). An Introduction to the Family *Flavobacteriaceae.* In *The Prokaryotes*. 7th edn, Ch. 6.3, pp. 455–480. Edited by E. Stackebrandt, A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

Bernardet, J.-F. (2011). Family I. *Flavobacteriaceae* Reichenbach 1992b (Effective publication: Reichenbach 1989/2013.) emend. Bernardet, Segers, Vancanneyt, Berthe, Kersters and Vandamme 1996, 145 emend, Bernardet, Nakagawa and Holmes 2002, 1057. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Volume 4: The *Bacteroidetes*, *Spirochaetes*, *Tenericutes* (*Mollicutes*), *Acidobacteria*, *Fibrobacteres*, *Fusobacteria*, *Dictyoglomi*, *Gemmatimonadetes*, *Lentisphaerae*, *Verrucomicrobia*, *Chlamydiae*, and *Planctomycetes* pp. 106–111. Edited by N. R. Krieg, J. T. Staley, D. R. Brown, B. P. Hedlund, B. J. Paster, N. L. Ward, W. Ludwig & W. B. Whitman. New York: Springer.

Bernardet, J.-F., Hugo, C. J. & Bruun, B. (2011). *Genus X*. **Chryseobacterium** *Vandamme, Bernardet, Segers, Kersters and Holmes 1994, 829^{VP}*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Volume 4: The *Bacteroidetes*, *Spirochaetes*, *Tenericutes* (*Mollicutes*), *Acidobacteria*, *Fibrobacteres*, *Fusobacteria*, *Dictyoglomi*, *Gemmatimonadetes*, *Lentisphaerae*, *Verrucomicrobia*, *Chlamydiae*, and *Planctomycetes* pp. 180–196. Edited by N. R. Krieg, J. T. Staley, D. R. Brown, B. P. Hedlund, B. J. Paster, N. L. Ward, W. Ludwig & W. B. Whitman. New York: Springer.

BIOLOG Inc. (2008). Biolog Omnilog Gen III System User Guide.

Blickstad, E., Enfors, S.-O. & Molin, G. (1981). Effect of hyperbaric carbon dioxide pressure on the microbial flora of pork stored at 4 °C or 14 °C. *J Appl Bacteriol* **50**, 493–504.

Blickstad, E. & Molin, G. (1983). Carbon dioxide as a controller of the spoilage flora of pork, with special reference to temperature and sodium chloride. *J Food Protect* **46**, 756–763.

Bloch, K. C., Nadarajah, R. & Jacobs, R. (1997). *Chryseobacterium meningosepticum*: an emerging pathogen among immunocompromised adults. *Medicine* (*Baltimore*) **76**, 30–41.

Bochner, B. R. (1989). Sleuthing out bacterial identities. *Nature* **339**, 157–158.

Bochner, B. R. (2003). New technologies to assess genotype-phenotype relationships. *Nat Rev Genet* **4**, 309–314.

Bochner, B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiol Rev* **33**, 191–205.

Bochner, B. R., Gadzinski, P. & Panomitros, E. (2001). Phenotype MicroArray for high-throughput phenotypic testing and assay of gene function. *Genome Res* **11**, 1246–1255.

Bochner, B. R., Giovannetti, L. & Viti, C. (2008). Important discoveries from analysing bacterial phenotypes. *Mol Microbiol* **70**, 274–280.

Bockle, B., Galunski, B. & Muller, R. (1995). Characterisation of a keratinolytic serine protease from *Streptomyces pactum* DSM40530. *Appl Environ Microbiol* **61**, 3705–3710.

Bockle, B. & Muller, R. (1997). Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feathers. *Appl Environ Microbiol* **63**, 790–792.

Bowman, J. P., McCammon, S. A., Brown, J. L., Nichols, D. S. & McMeekin, T. A. (1997). *Psychroserpens burtonensis* gen. nov., sp. nov., and *Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. *Int J Syst Bacteriol* **47**, 670–677.

Bowman, J. P., McCammon, S. A., Lewis, T., Skerratt, J. H., Brown, J. L., Nichols, D. S. & McMeekin, T. A. (1998). *Psychroflexus torquis* gen. nov., sp. nov., a pschrophilic species from Antactic sea ice, and reclassification of *Flavobacterium gondwanense* (Dobson *et al.*, 1993) as *Psychroflexus gondwanense* gen. nov. comb. nov. *Microbiol* **144**, 1601–1609.

Brandelli, A. & Riffel, A. (2005). Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers. *E J Biotechnol* **8**(1), 35–42.

Britz T. J. & Kriel J. B. (1973). Short-term preservation of bacterial stock cultures. *South Afr J Dairy Technol* **5**, 209–212.

Brummer, I. H. M., Fehr, W. & Wagner-Döbler, I. (2000). Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. *Appl Environ Microbiol* **66**, 3078–3082.

Buck, J. D. (1982). Non-staining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44,** 992–993.

Busse, J. & Auling, G. (1988). Polyamine pattern as a chemotaxonomic marker within the proteobacteria. *Syst Appl Microbiol* **11**, 1–8.

Campbell, S., Harada, R. M. & Li, Q. X. (2008). *Chryseobacterium arothri* sp. nov., isolated from the kidneys of a pufferfish. *Int J Syst Evol Microbiol* **58**, 290–293.

Casarin, F., Cladera-Olivera, F. & Brandelli, A. (2008). Use of poultry byproduct for production of keratinolytic enzymes. *Food Bioprocess Technol* **1**, 301–305.

Cascio, A., Stassi, G., Costa, G. B., Crisafulli, G., Rulli, I., Ruggeri, C. & Iaria, C. (2005). *Chryseobacterium indologenes* bacteraemia in a diabetic child. *J Med Microbiol* **54**, 677–680.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Chesson, A. (1980). Maceration in relation to the post-harvest handling and processing of plant material. *J Appl Bacteriol* **48,** 1–45.

Cho, S.-H., Lee, K. S., Shin, D.-S., Han, J.-H., Park, K. S., Lee, C. H., Park, K. H. & Kim, S. B. (2011). Four new species of *Chryseobacterium* from the rhizosphere of coastal sand dune plants, *Chryseobacterium elymi* sp. nov., *Chryseobacterium hagamense* sp. nov., *Chryseobacterium lathyri* sp. nov. and *Chryseobacterium rhizosphaerae* sp. nov. *Int J Syst Evol Microbiol* **61**, 1–3.

Choi, J. M. & Nelson, P. V. (1996). Developing a slow-release nitrogen fertilizer from organic sources. 2. Using poultry feathers. *J Am Soc Horticultural Sci* **126**(4), 634–638.

Christen, G. L. & Marshal, R. T. (1984). Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media. *J Dairy Sci* **67**, 1680–1687.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* **17**, 840–862.

Clayton, R. A., Sutton, G., Hinkle, P. S. Jr., Bult, C. & Fields, C. (1995). Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. *Int J Syst Bacteriol* **45**, 595–599.

Coenye, T., Gevers, D., Van de Peer, Y., Vandamme, P. & Swings, J. (2005). Towards a prokaryotic genomic taxonomy. *FEMS Microbiol Rev* **29**, 147–167.

Colwell, R. R. (1970). Polyphasic taxonomy of the genus *Vibrio*: numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and related *Vibrio* species. *J Bacteriol* **104,** 410–433.

Cottrell, M. T. & Kirchman, D. L. (2000) Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence *in situ* hybridization. *Appl Environ Microbiol* **66**, 5116–5122.

Coultate, T. P. (1984). *Food: The Chemistry of its Components*. London: Royal Society of Chemistry, Paperbacks.

Cowan, S. T. (1974). *Cowan and Steel's Manual for the Identification of Medical Bacteria* 2nd edn. Cambridge: University Press.

Czekajło, K. U., Giedrys-Kalemba, S. & Mędrala, D. (2006). Phenotypic and genotypic characteristics of *Pseudomonas aeruginosa* strains isolated from hospitals in the north-west region of Poland. *Polish J Microbiol* **55**, 103–112.

Dalev, P. & Neitchev, V. (1991). Reactive alkaline protease to keratin and collagen containing substances. *Appl Biochem Biotechnol* **27**(2), 131–138.

de Beer, H. (2005). *A Taxonomic Study of Chryseobacterium Species in Meat*. Ph.D. Thesis. University of the Free State, Bloemfontein, South Africa.

De Beer, H. & Hugo, C. J. (2010). Potential sources of *Chryseobacterium* contamination during poultry processing: A pilot study. *J New Generation Sci* **8**(3), 20–28.

de Beer, H., Hugo, C. J., Jooste, P. J., Vancanneyt, M., Coenye, T. & Vandamme, P. (2006). *Chryseobacterium piscium* sp. nov., isolated from fish of the South Atlantic Ocean off South Africa. *Int J Syst Evol Microbiol* **56**, 1317–1322.

de Beer, H., Hugo, C. J., Jooste, P. J., Willems, A., Vancanneyt, M., Coenye, T. & Vandamme, P. A. R. (2005). *Chryseobacterium vrystaatense* sp. nov., isolated from raw chicken in a chicken-processing plant. *Int J Syst Evol Microbiol* **55**, 2149–2153.

Deeth, H. C., Khusniati, T., Datta, N. & Wallace, R. B. (2002). Spoilage patterns of skim and whole milks. *J Dairy Res* **69**, 227–241.

De Ley, J., Cottoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

De Toni, C. H., Richter, M. F., Chagas, J. R., Henriques, J. A. P. & Termignoni, C. (2002). Purification and characterization of an alkaline serine endopeptidase from a feather-degrading *Xanthomonas maltophila* strain. *Can J Microbiol* **48,** 342–348.

Dhawan, D., Kaur, S. & Jagdeep, J. (2008). Microbial mannases: An overview of production and applications. http://www.redorbit.com/news/science/1208643/microbial_mannases_an__overview_of_production_and_applications/ Accessed: on 25 January, 2012.

Drancourt, M., Bollett, C., Carlioz, A., Martelin, R., Gayral, J.-P. & Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol* **38**, 3623–3630.

Drummond, A. J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T. & Wilson, A. (2009). Geneious v 4.8. Available from http://www.geneious.com/

Ebbing, D. D. & Gammon, S. D. (1999). *General Chemistry*, 6th edn. Houghton Mifflin Company, Boston, U. S. A.

Eck, R. V. & Dayhoff, M. O. (1966). *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Silver Springs, Maryland.

Eggum, B. O. (1970). Evaluation of protein quality of feather meal under different treatments. *Acta Agric Scand* **20**, 230–234.

Enfors, S.-O., Molin, G. & Ternström, A. (1979). Effect of packaging under carbon dioxide, nitrogen or air on the microbial flora of pork stored at 4 °C. *J Appl Bacteriol* **47**, 197–208.

Engelbrecht, K. (1992). *Spoilage Bacteria Associated with Cape Hake (*Marlussins carpensis *and* Merhissins paradoxus*) and Other Edible South Atlantic Species*. M.Sc. Thesis. University of the Orange Free State, Bloemfontein, South Africa.

Engelbrecht, K., Jooste, P. J. & Prior, B. A. (1996). Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. *S A J Food Sci Nut* **8**(2), 66–71.

Erichsen, I. & Molin, G. (1981). Microbial flora of normal and high pH beef stored at 4 °C in different gas environments. *J Food Protect* **44**, 866–869.

Euzéby, J. P. (2012a). *List of Prokaryotic Names with Standing in Nomenclature*. http://www.bacterio.cict.fr/f/flavobacteriaceae.html (Accessed on 17 January 2012).

Euzéby, J. P. (2012b). *List of Prokaryotic Names with Standing in Nomenclature*. http://www.bacterio.cict.fr/c/chryseobacterium.html (Accessed on 17 January 2012).

FAO. (2008a). Organic wastes. http://www.fao.org/ag/AGA/AGAP/FRG/APH132/chap6.htm (Accessed on 03 March 2009).

FAO. (2008b). Important information on the feeding of animal by-products. http://www.fao.org/waicent/faoinfo/agricult/aga/AGAP/FRG/AFRIS/ DATA/326.htm (Accessed on 03 March 2009).

Fodor, S. A., Rava, R. P., Huang, X. C., Pease, A. C., Holmes, C. P. & Adams, C. L. (1993). Multiplexed biochemical assays with biological chips. *Nature* **364**, 555–556.

Forsythe, S. J. (2000). *The Microbiology of Safe Food*. London: Blackwell Science.

Frazier, W. C. 1988. Part 1: Foods and microorganisms. In: *Food Microbiology*. ed. E. Dallinger. McGraw-Hill, New York, pp. 76–79.

Friedrich, A. B. & Antranikian, G. (1996). Keratin degradation by *Fervidobacterium pennavorans,* a novel thermophilic anaerobic species of the order thermotogales. *Appl Environ Microbiol* **62,** 2875–2882.

Fries, R. & Graw, C. (1999). Water and air in two poultry processing plants' chilling facilities - a bacteriological survey. *Br Poultry Sci* **40**, 52–58.

Gallego, V., Garcia, M. T. & Ventosa, A. (2006). *Chryseobacterium hispanicum* sp. nov., isolated from the drinking water distribution system of Sevilla, Spain. *Int J Syst Evol Microbiol* **56**, 1589–1592.

Garbutt, J. (1997). Food-borne disease and food poisoning. In *Essentials of Food Microbiology*, pp. 135–181. London: Arnold.

García López, M.-L., Prieto, M. & Otero, A. (1998). The physiological attributes of Gram-negative bacteria associated with spoilage of meat and meat products. In *The Microbiology of Meat and Poultry*, pp. 1–34. Edited by A. Davies & R. Board. London: Blackie Academic and Professional.

García López, M.-L., Santos, J.-A. & Otero, A. (1999). *Flavobacterium*. In *Encyclopedia of Food Microbiology*, pp. 820–824. Edited by R. K. Robinson. San Diego: Academic Press.

Gassessse, A., Kaul, R. H., Gashe, B. A. & Mattiasson, B. (2003). Novel alkaline proteases from alkalophilic bacteria grown on chicken feathers. *Enzyme Microb Technol* **32**, 519–524.

Georgnaras, I. & von Holy, A. (2000). Bacterial counts associated with poultry processing at different sampling times. *J Basic Microbiol* **40**, 343–349.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L. & Swings, J. (2005). Opinion: Re-evaluating prokaryotic species. *Nat Rev Microbiol* **3**, 733–739.

Gevers, D., Dawyndt, P., Vandamme, P., Willems, A., Vancanneyt, M., Swings, J. & De Vos, P. (2006). Stepping stones towards a new prokaryotic taxonomy. *Phil Trans Royal Soc Biol* **361**, 1911–1916.

Gillis, M., Vandamme, P., De Vos, P., Swings, J. & Kersters, K. (2001). Polyphasic taxonomy. In *Bergey's Manual of Systematic Bacteriology*. 2nd edn. pp. 43–48. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. Berlin: Springer.

Gradisar, H., Kern, S. & Friedrich, J. (2000). Keratinase of *Doratomyces microsporus*. *Appl Microbiol Biotechnol* **53**, 196–200.

Gram, L. & Dalgaard, P. (2002). Fish spoilage bacteria – problems and solutions. *Cur Solutions Biotechnol* **13**(3), 262–266.

Grimont, P. A. D., Popoff, M. Y., Grimont, F., Coynault, C. & Lemelin, M. (1980). Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr Microbiol* **4,** 325–330.

Gupta, R. S. (2000). The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol Rev* **24**, 367–402.

Gupta, R. & Ramnani, P. (2006). Microbial keratinases and their prospective applications: an overview. *Appl Microbiol Biotechnol* **70**, 21–33.

Haack, S. K. & Breznak, J. A. (1993). *Cytophaga xylanolytica* sp. nov., a xylan degrading anaerobic gliding bacterium. *Arch Microbiol* **159**, 6–15.

Hamada, J. S. (1994). Deamidation of food proteins to improve functionality. *Crit Rev Food Sci Nutr* **34,** 283–292.

Hamana, K. & Matsuzaki, S. (1991). Polyamine distributions in the *Flavobacterium-Cytophaga-Sphingobacterium* complex. *Can J Microbiol* **37**, 885–888.

Hantsis-Zacharov, E. & Halpern, M. (2007). *Chryseobacterium haifense* sp. nov., a psychrotolerant bacterium isolated from raw milk. *Int J Syst Evol Microbiol* **57**, 2344–2348.

Hantsis-Zacharov, E., Senderovich, Y. & Halpern, M. (2008a). *Chryseobacterium bovis* sp. nov., isolated from raw cow's milk. *Int J Syst Evol Microbiol* **58**, 1024–1028.

Hantsis-Zacharov, E., Shaked, T., Senderovich, Y. & Halpern, M. (2008b). *Chryseobacterium oranimense* sp. nov., a psychrotolerant, proteolytic and lipolytic bacterium isolated from raw cow's milk. *Int J Syst Evol Microbiol* **58**, 2635–2639.

Hanzawa, N., Kanai, S., Katsuta, A., Nakagawa, Y. & Yamasato, K. (1995). 16S rDNA-based phylogenetic analysis of marine favobacteria. *J Mar Biotechnol* **3**, 111–114.

Harrigan, F. W. & Park, R. W. A. (1991). *Making Safe Food: A Management Guide for Microbial Quality*. pp. 12–19. London: Academic Press.

Hendrie, M. S., Mitchel, T. G. & Shewan, J. M. (1969). The identification of yellow pigmented rods. In *Identification Methods for Microbiologists*; Part B, pp. 67–77. Edited by B. M. Gibbs & D. A. Shapton. London: Academic Press.

Herzog, P., Winkler, I., Wolking, D., Kämpfer, P. & Lipski, A. (2008). *Chryseobacterium ureilyticum* sp. nov., *Chryseobacterium gambrini* sp. nov., *Chryseobacterium pallidum* sp. nov. and *Chryseobacterium molle* sp. nov., isolated from beer-bottling plants. *Int J Syst Evol Microbiol* **58**, 26–33.

Hinton, A., Cason, J. A. & Ingram, K. D. (2002). Enumeration and identification of yeasts associated with commercial poultry processing and spoilage of refrigerated broiler carcasses. *J Food Protect* **65**, 993–998.

Hinton, A., Cason, J. A. & Ingram, K. D. (2004). Tracking spoilage bacteria in commercial poultry processing and refrigerated storage of poultry carcases. *Int J Food Microbiol* **91**, 155–165.

Hirsch, P., Ludwig, W., Hethke, C., Sittig, M., Hoffmann, B. & Gallikowski, C. A. (1998). *Hymenobacter roseosalivarius* gen. nov., sp. nov. from continental Antarctic soils and sandstone: bacteria of the *Cytophaga-Flavobacterium-Bacteroides* line of phylogenetic descent. *Syst Appl Microbiol* **21**, 374–383.

Holmes, B., Lapage, S.P. and Malnick, H. (1975). Strains of *Pseudomonas putrefaciens* from clinical material. *J Clin Pathol* **28,** 149–155.

Holmes, B., Snell, J. J. S. & Lapage, S. P. (1978). Revised description, from clinical strains, of *Flavobacterium breve* (Lustig) Bergey *et al*. 1923 and proposal of the neotype strain. *Int J Syst Bacteriol* **28**, 201–208.

Holmes, B. & Owen, R. J. (1979). Proposal that *Flavobacerium breve* be substituted as the type species of the genus in place of *Flavobacterium aquatile* and emended description of the genus *Flavobacterium*: status of the named species of *Flavobacterium*. *Int J Syst Bacteriol* **29**, 416–426.

Holmes, B., Owen, R. J. & McMeekin, T. A. (1984a). Genus *Flavobacterium* Bergey, Harrison, Breed, Hammer and Huntoon 1923, 97^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 353–361. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins Co.

Holmes, B., Owen, R. J., Steigerwalt, A. G. & Brenner, D. J. (1984b). *Flavobacterium gleum*, a new species found in human clinical specimens. *Int J Syst Bacteriol* **34**, 21–25.

Holmes, B., Steigerwalt, A. G., Weaver, R. E. & Brenner, D. J. (1986a). *Weeksella virosa* gen. nov., sp. nov. (formerly group IIf), found in human clinical specimens. *Syst Appl Microbiol* **8**, 185–190.

Holmes, B., Steigerwalt, A. G., Weaver, R. E. & Brenner, D. J. (1986b). *Weeksella zoohelcum* sp. nov. (formerly group IIj), from human clinical specimens. *Syst Appl Microbiol* **8**, 191–196.

Holmes, B. (1987). Identification and distribution of *Flavobacterium meningosepticum* in clinical material. *J Appl Bacteriol* **62**, 29–41.

Holmes, B. (1992). The genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*. In *The Prokaryotes*, 2nd edn, vol. 4, pp. 3620–3630. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. Berlin: Springer.

Holmes, B. (1993). Recent developments in *Flavobacterium* taxonomy. In *Advances in the Taxonomy and Significance of Flavobacterium, Cytophaga and Related Bacteria*. pp. 6–15. Edited by P. J. Jooste. University of the Orange Free State Press. Bloemfontein, South Africa.

Holmes, B. (1997). International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria, min of the meetings, 4 to 6 July 1994, Prague, Czech Republic. *Int J Syst Bacteriol* **47**, 593–594.

Hoque, S. N., Aubert, D., Naas, T. & Nordmann, P. (2001). *Chryseobacterium* (*Flavobacterium*) *meningosepticum* outbreak associated with colonization of water tap in neonatal intensive care unit. *J Hosp Infect* **47**, 188–192.

Hugo, C. J. (1997). *A Taxonomic Study of the Genus Chryseobacterium from Food and Environmental Sources*. Ph.D. thesis, Bloemfontein: University of the Orange Free State.

Hugo, C. J. & Jooste, P. J. (1997). Preliminary differentiation of food strains of *Chryseobacterium* and *Empedobacter* using multilocus enzyme electrophoresis. *Food Microbiol* **14**, 133–142.

Hugo, C. J. & Jooste, P. J. (2003). Culture media for genera in the family *Flavobacteriaceae*. In *Handbook of Culture Media for Food Microbiology*, Vol 37, pp. 355–367. Edited by J. E. L. Corry, G. D. W. Curtis & R. M. Baird. Amsterdam: Elsevier.

Hugo, C. J. & Jooste, P. J. (2012). Culture media for food associated genera in the family *Flavobacteriaceae*. In *Handbook of Culture Media for Food Microbiology*, Vol 37, pp. 355–367. Edited by J. E. L. Corry, G. D. W. Curtis & R. M. Baird. Amsterdam: Elsevier.

Hugo, C. J., Jooste, P. J., Segers, P., Vancanneyt, M. & Kersters, K. (1999). A polyphasic taxonomic study of *Chryseobacterium* strains isolated from dairy sources. *Syst Appl Microbiol* **22**, 586–595.

Hugo, C. J., Segers, P., Hoste, B., Vancanneyt, M. & Kersters, K. (2003). *Chryseobacterium joostei* sp. nov., isolated from the dairy environment. *Int J Syst Evol Microbiol* **53**, 771–777.

Humphry, D. R., George, A., Black, G. W. & Cummings, S. P. (2001). *Flavobacteria frigidarium* sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. *Int J Syst Evol Microbiol* **51**, 1235–1243.

Huss, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Im, W. T., Yang, J. E., Kim, S. Y. & Yi, T. H. (2011). *Chryseobacterium ginsenosidimutans* sp. nov., a bacterium with ginsenoside-converting activity isolated from soil of a *Rhus vernicifera*-cultivated field. *Int J Syst Evol Microbiol* **61**, 1430–1435.

Ivanova, E. P., Nedashkovskaya, O. I., Chun, J., Lysenko, A., Frolova, G. M., Svetashev, V. I., Vysotskii, M. V., Mikhailov, V. V., Huq, A. & Colwell, R. R. (2001). *Arenibacter* gen. nov., new genus of the family *Flavobacteriaceae* and the description of a new species, *Arenibacter latericius* sp. nov. *Int J Syst Evol Microbiol* **51**, 1987–1995.

Jackson, T. C., Acuff, G. R., Vanderzant, C., Sharp, T. R. & Savell, J. W. (1992). Identification and evaluation of volatile compounds of vacuum and modified atmosphere packaged beef strip loins. *Meat Sci* **31**, 175–190.

Jackson, T. C., Acuff, G. R. & Dickson, J. H. (1997). Meat, poultry and sea food. In *Food Microbiology: Fundamentals and Frontiers*. pp. 83–97. Edited by M. P. Doyle, L. R. Beuchat & J. M. Thomas. Goettingen, Germany: American Society for Microbiology.

Jay, J. M. (1992). *Modern Food Microbiology*, 4th edn. New York: Van Nostrand Reinhold.

Jay, J. M., Vilai, J. P. & Hughes, M. P. (2003). Profile and activity of bacterial biota of ground beef held from freshness to spoilage at 5-7^{o}C. *Int J Food Microbiol* **81**(2), 105–111.

Jodal, I., Kandra, I. L., Harangi, J., Nnisi, P. & Szejtli, J. (1984). Enzymatic degradation of cyclodextrins; Preparation and application of their fragments. *J Incl Phenom Microcyclic Chem* **2**(3–4), 877–884.

Johansen, J. E., Nielsen, P. & Sjoholm, C. (1999). Description of *Cellulophaga balitica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and

reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 1231–1240.

Johansen, J. E. & Binnerup, S. J. (2002). Contribution of *Cytophaga*-like bacteria to the potential of turnover of carbon, nitrogen and phosphorus by bacteria in the rhizosphere of barley (*Hordeum vulgare* L.). *Microb Ecol* **43**, 298–306.

Joshi, S. G., Tejashwini, M. M., Revati, N., Sridevi, R. & Roma, D. (2007). Isolation and characterization of a feather degrading bacterium. *Int J Poultry Sci* **6**(9), 689–693.

Jooste, P. J. (1985). *The Taxonomy and Significance of Flavobacterium-Cytophaga Strains from Dairy Sources.* Ph.D. thesis, Bloemfontein: University of the Orange Free State.

Jooste, P. J., Britz, T. J. & Lategan, P. M. (1986). The prevalence and significance of *Flavobacterium* strains in commercial salted butter. *Milchwissenschaft* **41**, 69–73.

Jooste, P. J. & Hugo, C. J. (1999). The taxonomy, ecology and cultivation of bacterial genera belonging to the family *Flavobacteriaceae*. *Int J Food Microbiol* **53**(2–3), 81–84.

Joung, Y. & Joh, K. (2011). *Chryseobacterium yonginense* sp. nov., isolated from a mesotrophic artificial lake. *Int J Syst Evol Microbiol* **61**, 1413–1417.

Jung, S.-Y., Kang, S.-J., Lee, M.-H., Lee, S.-Y., Oh, T.-K. & Yoon, J.-H. (2005). *Gaetbulibacter saemankumensis* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a tidal flat sediment in Korea. *Int J Syst Evol Microbiol* **55**, 1845–1849.

Kämpfer, P., Dreyer, U., Neef, A., Dott, W. & Busse, H. J. (2003). *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **53**, 93–97.

Kämpfer, P., Lodders, N., Vaneechoutte, M. & Wauters, G. (2009a). Transfer of *Sejongia antarctica*, *Sejongia jeonii* and *Sejongia marina* to the genus *Chryseobacterium* as *Chryseobacterium antarcticum* comb. nov., *Chryseobacterium jeonii* comb. nov. and *Chryseobacterium marinum* comb. nov. *Int J Syst Evol Microbiol* **59**, 2238–2240.

Kämpfer, P., Vaneechoutte, M., Lodders, N., de Baere, T., Avesani, V., Janssens, M., Busse, H.-J. & Wauters, G. (2009b). *Chryseobacterium anthropi* sp. nov., to accommodate clinical isolates biochemically similar to *Kaistella koreensis* and *Chryseobacterium haifense,* proposal to reclassify *Kaistella koreensis* as *Chryseobacterium koreense* comb. nov. and emended description of the genus *Chryseobacterium*. *Int J Syst Evol Microbiol* Papers in Press. Published July 21, 2009 as doi:10.1099/ijs.0.008250-0.

Kämpfer, P., Vaneechoutte, M. & Wauters, G. (2009c). *Chryseobacterium arothri* Campbell *et al*., 2008 is a later heterotypic synonym of *Chryseobacterium hominis* Vaneechoutte *et al*., 2007. *Int J Syst Evol Microbiol* **59**, 695–697.

Kämpfer, P. (2010). The characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* **60**(1), 7.

Kämpfer, P., Arun, A. B., Young, C.-C., Chen, W.-M., Sridhar, K. R. & Rekha, P. D. (2010a). *Chryseobacterium arthrosphaerae* sp. nov., isolated from the faeces of the pill millipede *Arthrosphaera magna* Attems. *Int J Syst Evol Microbiol* **60,** 1765–1769.

Kämpfer, P., Chandel, K., Prasad, G. B. K. S., Shouche, Y. S. & Veer, V. (2010b). *Chryseobacterium culicis* sp. nov., isolated from the midgut of the mosquito *Culex quinquefasciatus*. *Int J Syst Evol Microbiol* **60**, 2387–2391.

Kämpfer, P., Fallschissel, K. & Avendano-Herrera, R. (2011). *Chryseobacterium chaponense* sp. nov., isolated from farmed Atlantic salmon (*Salmo salar*). *Int J Syst Evol Microbiol* **61**, 497–501.

Kelleher, B. P., Leahy, J. J., Heniham, A. M., O'Dwyer, T. F., Sutton, D. & Leahy, M. J. (2002). Advances in poultry litter disposal technology – a review. *Biores Technol* **83,** 27–36.

Kienzle, N., Muller, M. & Pegg, S. (2000). *Chryseobacterium* in burn wounds. *J Int Soc Burn Injuries* **27**(2), 179–182.

Kim, J. M., Lim, W. J. & Suh, H. J. (2001). Feather-degrading *Bacillus* species from poultry waste. *Process Biochem* **37**, 287–291.

Kim, K. K., Kim, M. K., Lim, J. H., Park, H. Y. & Lee, S. T. (2005a). Transfer of *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to *Elizabethkingia* gen. nov. as *Elizabethkingia meningoseptica* comb. nov. and *Elizabethkingia miricola* comb. nov. *Int J Syst Evol Microbiol* **55**, 1287–1293.

Kim, K. K., Bae, H.-S., Schumann, P. & Lee, S.-T. (2005b). *Chryseobacterium daecheongense* sp. nov., isolated from freshwater lake sediment. *Int J Syst Evol Microbiol* **55**, 133–138.

Kim, K. K., Lee, K. C., Oh, H. M. & Lee, J. S. (2008). *Chryseobacterium aquaticum* sp. nov., isolated from a water reservoir. *Int J Syst Evol Microbiol* **58**, 533–537.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

King, E. O. (1959). Studies on a group of previously unclassified bacteria associated with meningitis in infants. *Am J Clin Pathol* **31**, 241–247.

Kirchman, D. L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.

Kojima, M., Kanai, M. & Tominaga, M. (2006). Isolation and characterization of a feather degrading enzyme from *Bacillus psedofirmus* FA30-01. *Extremophiles* **10**, 229–235.

Korkmaz, H., Hur, H. & Dincer, S. (2004). Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste. *Annals Microbiol* **54**, 201–211.

Kreplak, L., Doucet, J., Dumas, P. & Briki, F. (2004). New aspects of the α-helix to β-sheet transition in stretched hard α-keratin fibers. *Biophys J* **87**, 640–647.

Kunert, J. (1989). Biochemical mechanism of keratin degradation by the actinomycete *Streptomyces fradiae* and the fungus *Microsporum gypseum*: a comparison. *J Basic Microbiol* **29,** 597–604.

Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum. Int J Syst Bacteriol* **38,** 358–361.

Langeveld, J. P. M., Wang, J. J., Van de Wiel, D. F. M., Shih, G. C., Garssen, G. J., Bossers, A. & Shih, J. C. H. (2003). Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. *J Infect Dis* **188**, 1782–1789.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**(21), 2947–2948.

Layne, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Meth Enzymol* **10**, 447–455.

Lee, D. H., Kahng, H. Y., Lee, Y. S., Jung, J. S., Kim, J. M., Chung, B. S., Park, S. K. & Jeon, C. O. (2009). *Jejuia pallidilutea* gen. nov., sp. nov., a new member of the family *Flavobacteriaceae* isolated from seawater. *Int J Syst Evol Microbiol* **59**, 2148–2152.

Lee, G. G., Ferket, P. R. & Shih, J. H. (1991). Improvement of feather digestibility by bacterial keratinase as a feed additive. *FASEB J* **59**, 1312.

Lee, K., Lee, H. K., Choi, T. H. & Cho, J. C. (2007). *Sejongia marina* sp. nov., isolated from Antarctic seawater. *Int J Syst Evol Microbiol* **57**, 2917–2921.

Lei, X.-H. & Bochner, B. (2008). Phenotype MicroArray analysis of the metabolism of *Helicobacter pylori* (Abstract). *Spring Meeting, Soc Gen Microbiol*, Edinburgh.

Leisner, J. J. & Gram, L. (1999). Spolage of fish. In *Encyclopedia of Food Microbiology*. pp. 814. Edited by R. K. Robinson. San Diego: Academic Press.

Lijnen, H. R., van Hoef, B., Ugwu, F., Collen, D. & Roelants, I. (2000). Specific proteolysis of human plasminogen by a 24 kDa endopeptidase from a novel *Chryseobacterium* sp. *Biochem* **39,** 479–488.

Lopez, M. J., Nichols, N. N., Dien, B. S., Moreno, J. & Bothast, R. J. (2004). Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. *Appl Microbiol Biotechnol* **64**, 125–131.

Louws, F. J., Fulbright, D. W., Stephens, C. T. & de Bruijn, F. J. (1995). Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *Vesicatoria. Phytopath* **85**, 528–836.

Lupski, J. R. & Weinstock, G. M. (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* **174**, 4525–4529.

MacFaddin, J. F. (1980). *Biochemical Tests for Identification of Medical Bacteria* 2nd edn. Baltimore: Williams and Wilkins Company.

Madigan, M. T., Martinko, J. M. & Parker, J. (2000). Brock Biology of Microorganisms, 9th edn. Prentice Hall International, New Jersey.

Mai, T. & Conner, D. (2001). Identification of bacteria found in broiler deboning operations. *Poultry Sci* **80**, (Suppl. 1), 297.

Manczinger, L., Rozs, M., Vagvolgyi, C. & Kevei, F. (2003). Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. *World J Microbiol Biotechnol* **19**, 35–39.

Manuel, M. J., Miller, D. L., Frazier, K. S. & Hines, M. E. (2002). Bacterial pathogens isolated from cultured bullfrogs (*Rana castebeiana*). *J Vet Diag Invest* **15**(5), 431–433.

Manz, W., Wendt-Potthof, K., Neu, T. R., Szewzyk, U. & Lawrence, J. R. (1999). Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent *in situ* hybridization and confocal laser scanning microscopy. *Microbial Ecol* **37**, 225–237.

Maslow, J. N., Mulligan, M. E. & Arbeit, R. D. (1993). Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. *Clin Infect Dis* **17**, 153–164.

Matikeviciene, V., Masiliuniene, D. & Grigiskis, S. (2009). Degradation of keratin contaning wastes by bacteria with keratinolytic activity. Environment. Technology. Resources. *Proceedings of the 7th International Scientific and Practical Conference* **1**, 284–289.

McBain, A. J., Bartolo, R. G., Catrenich, C. E., Charbonneau, D., Ledder, R. G., Rickard, A. H., Symmons, S. A. & Gilbert, P. (2003). Microbial characterization of biofilms in domestic drains and establishment of stable biofilm microcosms. *J Appl Environ Microbiol* **69**, 177–185.

McMeekin, T. A. (1982). Microbial spoilage of meat. In *Developments in Food Microbiology*, Vol 1. pp. 1–40. Edited by R. Davies. London: Applied Science.

McMeekin, T. A. & Shewan, J. M. (1978). Taxonomic strategies for *Flavobacterium* and related genera. *J Appl Bacteriol* **34**, 321–332.

Mekalanos, J. J. (1992). Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol* **174**, 1–7.

Mesbah, M., Premachadran, U. & Franklin, M. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Michel, C., Matte-Tailliez, O., Kerouault, B. & Bernardet, J.-F. (2005). Resistance pattern and assessment of phenicol agents' minimum inhibitory concentration in multiple drug resistant *Chryseobacterium* isolates from fish and aquatic habitats. *J Appl Microbiol* **99**, 323–332.

MIDI Inc. (2001). MIDI Technical Note 101. MIDI Handbook.

Mitra, S., Matsuo, Y., Haga, T., Yasumoto-Hirose, M., Yoon, J., Kasai, H. & Yokota, A. (2009). *Leptobacterium flavescens* gen. nov., sp. nov., a marine member of the family *Flavobacteriaceae*, isolated from marine sponge and seawater. *Int J Syst Evol Microbiol* **59**, 207–212.

Molin, G. (2000). Modified atmospheres. In *The Microbiological Safety and Quality of Food.* Vol 1. pp. 214–229. Edited by B. M. Lund, T. C. Baird-Parker & G. W. Gould. Gaithersburg: Aspen.

Mora, R. R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Mora, R. R. (2006). DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In *Molecular Identification, Systematics, and Population Structure of Prokaryotes*. pp. 23–49. Edited by E. Stackebrandt. Berlin, Heidelberg: Springer-Verlag.

Morales, C. A., Gast, R. & Guard-Bouldin, J. (2006). Linkage of avian and reproductive truct tropism with sequence divergence adjacent to the 5S ribosomal subunit rrfH of *Salmonella enterica. FEMS Microbiol Lett* **264**, 48–58.

Morales, C. A., Porwollik, S., Frye, J. G., Kinde, H., McClelland, M. & Guard-Bouldin, J. (2005). Correlation of phenotype with the genotype of egg-contaminating *Salmonella enterica* serovar Enteritidis. *Appl Environ Microbiol* **71**, 4388–4399.

Mossel, D. A. A., Corry, J. E. L. & Baird, R. M. (1995). Evaluation of the efficacy of measures to ensure wholesomeness and quality of food by assessing compliance with reference values ('standards'). In *Essentials of the Microbiology of Foods. A textbook for Advanced Studies*. Ch 8, pp. 343–404. Chichester: John Wiley and Sons.

Mountney, G. J. & Gould, W. A. (1988). *Practical Food Microbiology and Technology*, 3rd edn. Van Nostrand Reinhold, New York.

Mountney, G. J. & Gould, W. A. (1998). Red meat and poultry. In *Practical Food Microbiology and Technology*. 3rd edn. pp. 157–167. New York: Van Nostrand Reinhold Company.

Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B. & Bernardet, J.-F. (1994). *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *Int J Syst Bacteriol* **44**, 447–453.

Murray, R. G. E., Brenner, D. J., Colwell, R. R., De Vos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stackebrandt, E. & Zavarzin, G. A. (1990). Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. *Int J Syst Bacteriol* **40**, 213–215.

Nagal, S. & Jain, P. C. (2010). Production of feather hydrolysate by *Elizabethkingia meningoseptica* KB042 MTCC 8360 in submerged fermentation. *Indian J Microbiol* **50**(1), doi: 10.1007/s12088-010-0014-0.

Nagal, S., Kango, N. & Jain, P. C. (2010). Production of alkaline protease from *Elizabethkingia meningoseptica* KB042 using chicken feathers. *Annals Microbiol* **60**, 629–635.

Nakagawa, Y. & Yamasato, K. (1996). Emendation of the genus *Cytophaga* and transfer of *Cytophaga agarovorans* and *Cytophaga salmonicolor* to *Marinilabilia* gen. nov.: phylogenetic analysis of the *Flavobacterium-Cytophaga* complex. *Int J Syst Bacteriol* **46**, 599–603.

Nam, G. W., Lee, D. W., Lee, H. S., Lee, N. J., Kim, B. C., Choe, E. A., Hwang, J. K., Suhartono, M. T. & Pyun, Y. R. (2002). Native feather degradation by *Fervidobacterium islandicum* AW-1, a newly isolated keratinase producing thermophilic anaerobe. *Arch Microbiol* **178,** 538–547.

Nedashkovskaya, O. I., Suzuki, M., Lee, J. S., Lee, K. C., Shevchenko, L. S. & Mikhailov, V. V. (2009). *Pseudozobellia thermophila* gen. nov., sp. nov., a bacterium of the family *Flavobacteriaceae*, isolated from the green alga *Ulva fenestrata*. *Int J Syst Evol Microbiol* **59**, 806–810.

Nottingham, P. M. (1982). Microbiology of carcass meats. In *Meat Microbiology*. pp. 13–66. Edited by M. H. Brown. London: Applied Science Publishers.

Nychas, G.-J. E. & Drosinos, E. H. (1999). Meat and poultry. In *Encyclopedia of Food Microbiology*, pp. 1253–1259. Edited by R. K. Robinson. San Diego: Academic Press.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007–4021.

Olive, D. M. & Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* **37**, 1661–1969.

Olofsson, T. C., Ahrné, S. & Molin, G. (2007). Composition of the bacterial population of refrigerated beef, identified with direct 16S rRNA gene analysis and pure culture technique. *Int J Food Microbiol* **118**, 233–240.

Olson, M. E., Gard, S., Brown, M., Hampton, R. & Morck, D. W. (1992). *Flavobacterium indologenes* infection in leopard frogs. Report on Wildlife and Laboratory Animals. *JVMA* **201**(11), 1767–1770.

On, S. L. W. & Holmes, B. (1991). Reproducibility of tolerance tests that are useful in the identification of campylobacteria. *J Clin Microbiol* **29**, 1785–1788.

On, S. L. W. & Holmes, B. (1992). Assessment of enzyme detection tests useful in identification of campylobacteria. *J Clin Microbiol* **30**, 746–749.

Onifade, A. A., Al-Sane, N. A., Al-Musallam, A. A. & Al-Zarban, S. (1998). Potentials for biotechnological applications of keratin degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Biores Technol* **66**, 1–11.

Papadopolous, M. C., El Boushy, A. R., Roodbeen, A. E. & Ketelaars, E. H. (1986). Effects of processing time and moisture content on amino acid composition and nitrogen characteristics of feather meal. *Anim Feed Sci Technol* **14**, 279–290.

Park, M. S., Jung, S. R., Lee, M.-S., Kim, K. O., Do, J. O., Lee, K. H., Kim, S. B. & Bae, K. S. (2006). *Chryseobacterium soldanellicola* sp. nov. and

Chryseobacterium taeanense sp. nov. isolated from roots of sand dune plants. *Int J Syst Evol Microbiol* **56**, 433–438.

Park, S. C., Kim, M. S., Baik, K. S., Kim, E. M., Rhee, M. S. & Seong, C. N. (2008). *Chryseobacterium aquifrigidense* sp. nov., isolated from a water-cooling system. *Int J Syst Evol Microbiol* **58**, 607–611.

Parry, D. A. D. & North, A. C. T. (1998). Hard α–keratin intermediate filament chains: substructure of the N- and C-terminal domains and the predicted structure and function of the C-terminal domain soft type I and type II chains. *J Struct Biol* **122**, 67–75.

Pavlov, D., de Wet, D. C. M., Grabow, W. O. K. & Ehlers, M. M. (2004). Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *Int J Food Microbiol* **92**, 275–287.

Peng, F., Liu, M., Zhang, L., Dai, J., Luo, X., An, H. & Fang, C. (2009). *Planobacterium taklimakanense* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* that exhibits swimming motility, isolated from desert soil. *Int J Syst Evol Microbiol* **59**, 1672–1678.

Pissuwan, D. & Suntornsuk, W. (2001). Production of keratinase by *Bacillus* sp. FK 28 isolated in Thailand. *Kasetsart J* **35,** 171–178.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*. pp. 494–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester, U.K: John Wiley & Sons.

Prakash, O., Verma, M., Sharma, P., Kumar, M., Kumari, K., Singh, A., Kumari, H., Jit, S., Gupta, S. K., Khanna, M. & Lal, R. (2007). Polyphasic approach of bacterial classification – An overview of recent advances. *Indian J Microbiol* **47,** 98–108.

Quan, Z. X., Kim, K. K., Kim, M. K., Jin, L. & Lee, S. T. (2007). *Chryseobacterium caeni* sp. nov., isolated from bioreactor sludge. *Int J. Syst Evol Microbiol* **57**, 141–145.

Radianingtyas, H., Robinson, G. K. & Bull, A. T. (2003). Characterization of a soil-derived bacterial consortium degrading 4-chloroaniline. *Microbiology-SGM* **149**, 3279–3287.

Ramnani, P. & Gupta, R. (2007). Keratinases vis-à-vis conventional proteases and feather degradation. *World J Biotechnol* **23**, 1537–1540.

Ravenschlag, H. & Dworkin, M. (2001). Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). *App Environ Microbiol* **67**, 387–395.

Regnault, B., Grimont, F. & Grimont, P. A. (1997). Universal ribotyping method using a chemically labelled oligonucleotide probe mixture. *Res Microbiol* **148**, 649–659.

Reichenbach, H. (1989). Order I. *Cytophagales* Leadbetter 1974. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 2011–2013. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.

Reichenbach, H. (1992). *Flavobacteriaceae* fam. nov. In *Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSB*, List no. 41. *Int J Syst Bacteriol* **42**, 327–329.

Richard, C. & Kiredjian, M., (1995). Laboratory Methods for the Identification of Strictly Aerobic Bacilli. Paris: Intstitut Pasteur.

Riffel, A. & Brandelli, A. (2006). Keratinolytic bacteria isolated from feather waste. *Braz J Microbiol* **37**, 395–399.

Riffel, A., Brandelli, A., Bellato, C. M., Souza, G. H. M. F., Eberlin, M. N. & Tavares, F. C. A. (2007). Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. kr6. *J Biotechnol* **128**, 693–703.

Riffel, A., Lucas, F., Heeb, P. & Brandelli, A. (2003). Characterisation of a new keratinolytic bacterium that completely degrades native feather keratin. *Arch Microbiol* **179**, 258–265.

Rissen, S. & Antranikian, G. (2001). Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity. *Extremophiles* **5,** 399–408.

Rossello-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Rosseló-Mora, R. (2006). DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In *Molecular Identification, Systematics and Population Structure of Prokaryotes*, pp. 23–50. Edited by E. Stackebrandt. Berlin: Springer.

Russell, S. M., Fletcher, D. L. & Cox, N. A. (1996). Spoilage bacteria of fresh broiler chicken carcasses. *Poultry Sci* **75**, 2041–2047.

Sack, E. L. W., van der Wielen, P. W. J. J. & van der Kooij, D. (2011). *Flavobacterium johnsoniae* as a model organism for characterizing biopolymer utilization in oligotrophic freshwater environments. *Am Soc Microbiol* **77**, 6931–6938.

Sakai, T., Kimura, H. & Kato, I. (2002). A marine strain of *Flavobacteriaceae* utilizes brown seaweed fucoidan. *Mar Biotechnol* **4**, 399–405.

Sangalli, S. & Brandelli, A. (2000). Feather keratin hydrolysis by a *Vibrio* sp. strain kr2. *J Appl Microbiol* **89**, 735–743.

Santos, R. M. D., Firmino, A. A. P., Sa, C. M. & Felix, C. R. (1996). Keratinolytic activity of *Aspergillus fumigatus* Fresenius. *Curr Microbiol* **33**, 364–370.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.

Segers, P., Mannheim, W., Vancanneyt, M., De Brandt, K., Hinz, K.-H., Kersters, K. & Vandamme, P. (1993a). *Riemerella anatipestifer* gen. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic affiliation within the *Flavobacterium-Cytophaga* rRNA homology group. *Int J Syst Bacteriol* **43**, 768–776.

Segers, P., Vandamme, P., Steyn, P. L., Mannheim, W., Willekens, H., Bauwens, M., De Ley, J. & Kersters, K. (1993b). Phylogenetic studies of *Flavobacterium* and related organisms by DNA-rRNA hybridizations. In *Advances in the Taxonomy and Significance of Flavobacterium*, *Cytophaga and Related Bacteria*, pp. 129–136. Edited by P. J. Jooste. Bloemfontein, South Africa: University of the Orange Free State Press.

Shen, F.-T., Kämpfer, P., Young, C.-C., Lai, W.-A. & Arun, A. B. (2005). *Chryseobacterium taichungense* sp. nov., isolated from contaminated soil. *Int J Syst Evol Microbiol* **55**, 1301–1304.

Shewan, J. M. & McMeekin, T. A. (1983). Taxonomy and ecology of *Flavobacterium* and related bacteria. *Ann Rev Microbiol* **37**, 233–252.

Shih, J. C. H. (1993). Recent developments in poultry waste digestion and feather utilization – a review. *Poultry Sci* **72**, 1617–1620.

Shimomura, K., Kaji, S. & Hiraishi, A. (2005). *Chryseobacterium shigense* sp. nov., a yellow-pigmented, aerobic bacterium isolated from a lactic acid beverage. *Int J Syst Evol Microbiol* **55**, 1903–1906.

Sohn, J. H., Lee, J.-H., Yi, H., Chun, J., Bae, K. S., Ahn, T.-Y. & Kim, S. J. (2004). *Kordia algicida* gen. nov., sp. nov., an algicidal bacterium isolated from red tide. *Int J Syst Evol Microbiol* **54**(3), 675–680.

Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: Tarnished gold standards. *Microbiol Today* **33**, 152–155.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rossello-Mora, R., Swings, J., Truper, H. G., Vauterin, L., Ward, A. C. & Whitman, W. B. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA gene sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Strahan, B. L., Failor, K. C., Batties, A. M., Hayes, P. S., Cicconi, K. M., Mason, C. T. & Newman, J. D. (2011). *Chryseobacterium piperi* sp. nov., isolated from a freshwater creek. *Int J Syst Evol Microbiol* **61**, 2162–2166.

Suzuki, K., Goodfellow, M. & O'Donnell, A. G. (1993). Cell envelopes and classification. In *Handbook of New Bacterial Systematics*, pp. 195–250. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press.

Suzuki, M., Nakagawa, Y., Harayama, S. & Yamamoto, S. (2001). Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bacteria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *Int J Syst Evol Microbiol* **51**, 1639–1652.

Szoboszlay, S., Atzél, B., Kukolya, J., Tóth, E. M., Márialigeti, K., Schumann, P. & Kriszt, B. (2008). *Chryseobacterium hungaricum* sp. nov., isolated from hydrocarbon-contaminated soil. *Int J Syst Evol Microbiol* **58**, 2748–2754.

Tai, C.-J., Kuo, H.-P., Lee, F.-L., Chen, H.-K., Yokota, A. & Lo, C.-C. (2006). *Chryseobacterium taiwanense* sp. nov., isolated from soil in Taiwan. *Int J Syst Evol Microbiol* **56**, 1771–1776.

Takeuchi, M. & Yokota, A. (1992). Proposals of *Sphingobacterium faecium* sp. nov., *Sphingobacterium piscium* sp. nov., *Sphingobacterium heparinum* comb. nov., *Sphingobacterium thalpophilum* comb. nov., and two genospecies of the genus *Sphingobacterium*, and synonymy of *Flavobacterium yabuuchiae* and *Sphingobacterium. J Gen Appl Microbiol* **38,** 465–482.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbio Lett* **25**, 125–128.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tatum, H. W., Ewing, W. H. & Weaver, W. H. (1974). Miscellaneous Gram-negative bacteria. In *Manual of Clinical Microbiology*, pp. 270–294. Edited by E. H. Lenette, E. H. Spalding & J. P. Truant. Washington, D.C.: American Society for Microbiology.

Thomas, C. J. & McMeekin, T. A. (1980). Contamination of broiler carcass skin during commercial processing procedures: an electron microscopic study. *Appl Environ Microbiol* **40**, 133–144.

Thys, R. C. S., Lucas, F. S., Riffel, A., Heeb, P. & Brandelli, A. (2004). Characterization of a protease of a feather-degrading *Microbacterium* species. *Lett Appl Microbiol* **39**, 181–186.

Tindall, B. J. (1990a). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

Tindall, B. J. (1990b). Lipid composition *of Halobacterium lacusprofundi. FEMS Microbiol Lett* **66**, 199–202.

Tindall, B. J., Rossello-Mora, R., Busse, H.-J., Ludwig, W. & Kämpfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* **60**, 249–266.

Triyanto & Wakabayashi, H. (1999). Genotypic diversity of strains of *Flavobacterium columnare* from diseased fishes. *Fish Pathol* **34**(2), 65–71.

Vancanneyt, M., Segers, P., Torck, U., Hoste, B., Bernardet, J.-F., Vandamme, P. & Kersters, K. (1996). Reclassification of *Flavobacterium odoratum* (Stutzer 1929) strains to a new genus, *Myroides*, as *Myroides odoratus* comb. nov. and *Myroides odoratimimus* sp. nov. *Int J Syst Evol Microbiol* **46**, 926–932.

Vandamme, P., Bernardet, J.-F., Segers, P., Kersters, K. & Holmes, B. (1994a). New perspectives in the classification of the flavobacteria: Description of *Chryseobacterium* gen. nov., *Bergeyella* gen. nov., and *Empedobacter* nom. rev. *Int J Syst Evol Microbiol* **44(**4), 827–831.

Vandamme, P., Segers, P., Vancanneyt, M., Kersters, K., Falsen, E., Devriese, L. A., Bisgaard, M., Hinz, K.-H. & Mannheim, W. (1994b). *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from the avian respiratory tract. *Int J Syst Evol Microbiol* **44**, 24–37.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K. & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407–438.

Vaneechoutte, M., Kämpfer, P., de Baere, T., Avesani, V., Janssens, M. & Wauters, G. (2007). *Chryseobacterium hominis* sp. nov., to accommodate clinical isolates biochemically similar to CDC groups II-h and II-c. *Int J Syst Evol Microbiol* **57**, 2623–2628.

Van Trappen, S., Vandecandelaere, I., Mergaert, J. & Swings, J. (2004). *Gillisia limnaea* gen. no., sp. nov., a new member of the family *Flavobacteriaceae* isolated from a bacterial mat in Lake Fryxell, Antarctic. *Int J Syst Evol Microbiol* **54**(2), 445–448.

Van Wyk, E. R. (2008). *Virulence Factors and Other Clinically Relevant Characteristics of Chryseobacterium Species*. M.Sc. Thesis. University of the Free State. Bloemfontein, South Africa.

Venter, H., Osthoff, G. & Litthauer, D. (1999). Purification and characterization of a metalloprotease from *Chryseobacterium* indologenes Ix9a and determination of the amino acid specificity with electrospray mass spectrometry. *Prot Expr Purif* **15**, 282–295.

Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Meth Cell Mol Biol* **5**, 25–40.

Vidal, L., Christen, P. & Coello, M. N. (2000). Feather degradation by *Kocuria rosea* in submerged culture. *World J Microbiol Biotechnol* **16,** 551–554.

Voet, D. & Voet, J. G. (1995). Three-dimensional structure of proteins. In *Biochemistry*, 2nd edn, pp. 154–156. Edited by J. Stiefel. New York: Wiley.

Wang, S.-L., Hsu, W.-T., Liang, T.-W., Yen, Y.-H. & Wang, C.-L. (2008). Purification and characterization of three novel keratinolytic metalloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Biores Technol* **99**, 5679–5686.

Wang, X. & Parsons, C. M. (1997). Effect of processing systems on protein quality of feather meal and hog hair meals. *Poultry Sci* **76**, 491–496.

Wayne, L. G., Brenner, D. J., Colwell, R. R. Grimont, P. A. D., Kandler, P., Krichevski, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. & Truper, H. G. (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Weeks, O. B. & Breed, R. S. (1957). Genus III. *Flavobacterium*. In *Bergey's Manual of Determinative Bacteriology*, 7th edn. pp. 309. Edited by R. S. Breed, E. G. D. Murray & N. R. Smith. Baltimore: Williams & Wilkins.

Weeks, O. B. (1974). Genus *Flavobacterium* Bergey *et al*. 1923. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 357–364. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore, Williams & Wilkins.

Weeks, O. B. (1981). Preliminary studies of the pigments of *Flavobacterium breve* NCTC 11099 and *Flavobacterium odoratum* NCTC 11036. In *The Flavobacterium-Cytophaga Group*, pp. 108–114. Edited by H. Reichenbach & O. B. Weeks. Weinheim: Gesellschaft for Biotechnologische Forschung.

Welch, D. F. (1991). Applications of cellular fatty acid analysis. *Clin Microbiol Rev* **4,** 422–438.

Weon, H.-Y., Kim, B.-Y., Yoo, S.-H., Kwon, S.-W., Cho, Y.-H., Go, S.-J. & Stackebrandt, E. (2006). *Chryseobacterium wanjuense* sp. nov., isolated from greenhouse soil in Korea. *Int J Syst Evol Microbiol* **56**, 1501–1504.

Weon, H. Y., Kim, B. Y., Yoo, S. H., Kwon, S. W., Stackebrandt, E. & Go, S. J. (2008). *Chryseobacterium soli* sp. nov. and *Chryseobacterium jejuense* sp. nov., isolated from soil samples from Jeju, Korea. *Int J Syst Evol Microbiol* **58**, 470–473.

West, P. A. & Colwell, R. R. (1984). Identification and Classification of the *Vibrionaceae* – an overview. In *Vibrios* in the Environment, pp. 285-363. Edited by R. R. Colwell. New York: Wiley.

Whiteley, A. S. & Bailey, M. J. (2000). Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Appl Environ Microbiol* **66**, 2400–2407.

Williams, C. M., Richter, C. H., MacKenzie, J. M. & Shih, J. C. H. (1990). Isolation, identification and characterization of a feather degrading bacterium. *Appl Environ Microbiol* **56**, 1509–1515.

Williams, C. M., Lee, C. G., Garlich, J. D. & Shih, J. C. H. (1991). Evaluation of a bacterial feather fermentation product, feather-lysate, as a feed protein. *Poultry Sci* **70**, 85–94.

Woese, C. R., Stackebrandt, E., Macke, T. J. & Fox, G. E. (1985). A phylogenetic definition of the major eubacterial taxa. *Syst Appl Microbiol* **6**, 143–151.

Yabuuchi, E., Hashimoto, Y., Ezaki, T., Ido, Y. & Takeuchi, N. (1990). Genotypic and phenotypic differentiation of *Flavobacterium indologenes* Yabuuchi *et al*. 1983 from *Flavobacterium gleum* Holmes *et al*. 1984. *Microbiol Immunol* **34**, 73–76.

Yabuuchi, E., Kaneko, T., Yano, I., Moss, C. W. & Miyoshi, N. (1983). *Sphingobacterium* gen. nov., *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb. nov., *Sphingobacterium mizutae* sp. nov.,

and *Flavobacterium indologenes* sp. nov.: glucose-nonfermenting gram-negative rods in CDC groups IIk-2 and IIb. *Int J Syst Bacteriol* **33**, 580–598.

Yamaguchi, S. & Yokoe, M. (2000). A novel protein-deamidating enzyme from *Chryseobacterium proteolyticum* sp. nov., a newly isolated bacterium from soil. *Appl Environ Microbiol* **66**, 3337–3343.

Yamaguchi, S., Jeenes, D. & Archer, D. B. (2001). Protein glutaminase from *Chryseobacterium proteolyticum*, an enzyme that deamidates glutaminyl residues in proteins. *Eur J Biochem* **268,** 1410–1421.

Yamamoto, S. & Harayama, S. (1996). Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequences of *gyrB* genes and on the amino acids sequences of their products. *Int J Syst Bacteriol* **46**, 506–511.

Yamamura, S., Morita, Y., Hasan, Q., Rao, S. R., Murakami, Y., Yokoyama, K. & Tamiya, E. (2002). Characterization of a new keratin-degrading bacterium isolated from deer fur. *J Biosci Bioeng* **93,** 595–600.

Yamauchi, K., Yamauchi, A., Kusunoki, T., Khoda, A. & Konishi, Y. (1996). Preparations of stable aqueous solutions of keratins and physicochemical and biodegradational properties of films. *J Biomed Mat Res* **31**, 439–444.

Yi, H., Yoon, H. I. & Chun, J. (2005). *Sejongia antarctica* gen. nov., sp. nov., *Sejongia jeonii* sp. nov., isolated from the Antarctic. *Int J Syst Evol Microbiol* **55**, 423–426.

Yu, J. & Ward, O. (1996). Investigation of the biodegradation of pentachlorophenol by the predominant bacterial strains in a mixed culture. *Int Biodeter Biodegrad* **5,** 181–187.

Yoon, J. H., Kang, S. J. & Oh, T. K. (2007). *Chryseobacterium daeguense* sp. nov., isolated from wastewater of a textile dye works. *Int J Syst Evol Microbiol* **57**, 1355–1359.

Young, C. C., Kämpfer, P., Shen, F. T., Lai, W. A. & Arun, A. B. (2005). *Chryseobacterium formosense* sp. nov., isolated from the rhizosphere of *Lactuca sativa* L. (garden lettuce). *Int J Syst Evol Microbiol* **55**, 423–426.

Zerdani, I., Faid, M. & Malki, A. (2004). Feather wastes digestion by new isolated strains *Bacillus* sp. in Morocco. *Afr J Biotechnol* **3**, 67–70.

Zhao, Q., Bai, Y., Zhang, G., Zhu, S., Sheng, H., Sun, Y. & An, L. (2011). *Chryseobacterium xinjiangense* sp. nov., isolated from alpine permafrost. *Int J Syst Evol Microbiol* **61**, 1397–1401.

Zhou, Y., Dong, J., Wang, X., Huang, X., Zhang, K.-Y., Zhang, Y.-Q., Guo, Y.-F., Lai, R. & Li, W.-J. (2007). *Chryseobacterium flavum* sp. nov., isolated from polluted soil. *Int J Syst Evol Microbiol* **57**, 1765–1769.

CHAPTER 10

SUMMARY

Species of the genus *Chryseobacterium* (family *Flavobacteriaceae*) occur widely in clinical, environmental and industrial ecosystems. In the clinical environment, they are uncommon etiologic agents, but their infections may be serious in immunocompromised patients. They are often resistant to multiple antimicrobial agents making infections due to these organisms potentially difficult to treat. In the food environment, they are known to cause spoilage of foods such as canned products, milk and dairy products, fish, meat and poultry.

It is therefore necessary to be able to solve or anticipate and avert possible problems caused by *Chryseobacterium* species. This genus also has positive characteristics which include synthesis of a number of enzymes potentially useful in industry (e.g. keratinolytic enzymes), medicine (e.g. prion degradation) and turnover of organic matter in soil, water and sewage plants. Taxonomic studies are key to solving such problems by characterization and identification of such organisms. This also sets the foundation for investigation of the organism's beneficial roles and applications.

In this study, some *Chryseobacterium* strains isolated from poultry feather waste and raw chicken, were phenotypically characterized and identified using conventional tests and the BIOLOG Omnilog Gen II system. Phylogenies of seven selected isolates were determined using the 16S rRNA gene sequence analysis and they were further characterized using the BIOLOG Omnilog Gen III identification system. They fell into four taxonomic groups (Group 1: 1_F178; Group 2: 5_R23647; Group 3: 6_F141B and 7_F195; and Group 4: 8_R23573, 9_R23581 and 10_R23577) which did not show affiliation to any currently recognised type species of the genus *Chryseobacterium* suggesting that these groups were possible representatives of novel species.

Three selected strains (8_R23573, 9_R23581 and 10_R23577) were subjected to a polyphasic taxonomic study to determine their exact taxonomic identities. Results of the predominant respiratory menaquinone, fatty acid methyl esters and DNA base composition supported the affiliation of the strains to the genus *Chryseobacterium*. When subjected to DNA-DNA hybridization, the strains gave relatedness values of more than 81% among the three strains and less than 57% similarity between the strains and their two nearest phylogenetic neighbours *C. shigense* (DSM 17126^T) and *C. luteum* (LMG23785^T). A novel species emerged after a comparison of the phenotypic, chemotypic and genotypic results. The new species was described and the name *Chryseobacterium carnipullorum* sp. nov. was proposed.

Analysis using the BIOLOG Phenotype MicroArray (PM) system, revealed that *C. carnipullorum* has the potential to cause food spoilage mainly by utilizing carbohydrates, carboxylic acids and amino acids by producing metabolites which lead to souring, butyric spoilage defects, alkalinisation, bitter tastes and sulphide spoilage. The organism was also shown to have potential for biotechnological applications in food and stockfeed technology; coffee extraction, oil drilling and detergent industries; manufacture of artificial antigens and chemical diagnostic agents and release of oligosaccharides and oligopeptides in (ultra) oligotrophic freshwater environments.

It was found that the new species was able to produce extracellular keratinases that were able to extensively degrade chicken feather waste in 48 h. This has the potential of contributing toward solving the disposal problem which is experienced by the poultry industry that produces huge amounts of the recalcitrant feather waste as a by-product. Currently, a very small percentage of feather waste is steamed, treated chemically and ground to form dietary protein supplement for stockfeeds. Degradation of feathers using keratinolytic organisms is a more economical and environmentally friendly alternative. *Chryseobacterium carnipullorum* also has the potential for application in other biotechnological processes involving keratin hydrolysis. Hydrolysed feathers can be converted to fertilizers, glues, films, and they can be used as the source of rare amino acids, such as serine, cysteine and proline.

Keywords: *Flavobacteriaceae, Chryseobacterium*, *C. carnipullorum*, taxonomy, keratinase.

HOOFSTUK 10

OPSOMMING

Spesies van die *Chryseobacterium* genus (familie *Flavobacteriaceae*) kom wyd verspreid in kliniese, omgewings en industriële ekosisteme voor. In die kliniese omgewing is hulle seldsame etiologiese agente maar hulle infeksies kan ernstig wees vir immuun-onderdrukte pasiënte. Hulle is dikwels weerstandbiedend teen veelvoudige antimikrobiese middels wat veroorsaak dat infeksies wat deur hierdie organismes veroorsaak word, potensieël moeilik is om te behandel. In die voedselomgewing is hulle bekend daarvoor dat hulle bederf in ingemaakte produkte, melk en melkprodukte, vis, vleis en hoendervleis veroorsaak.

Dit is dus noodsaaklik om moontlike probleme wat deur *Chryseobacterium* veroorsaak word, op te los of te voorspel en sodoende die probleme te vermy. Hierdie genus het ook positiewe kenmerke wat insluit die sintese van ‘n aantal ensieme wat waardevol in die hoenderbedryf (bv. keratinolitiese ensieme) en in die geneeskunde (bv. prion afbraak) kan wees. Die organismes kan ook nuttig wees vir die verwerking van organiese materiaal in grond, water en rioolaanlegte. Taksonomiese studies is die sleutel tot die oplossing van sulke probleme deur die karakterisering en identifikasie van hierdie organismes. Sulke studies vorm ook die grondslag om die voordelige rolle en toepassings van die organismes te bepaal.

In hierdie studie is sommige *Chryseobacterium* stamme, geïsoleer vanaf hoendervere-afval en rou hoendervleis, fenotipies gekarakteriseer en geïdentifiseer met behulp van konvensionele toetse en die BIOLOG Omnilog Gen II sisteem. Die filogenetika van sewe gekose isolate is bepaal deur 16S rRNA geen-volgorde analise en is toe verder gekarakteriseer met die BIOLOG Omnilog Gen III identifikasie sisteem. Die isolate is in vier taksonomiese groepe verdeel (Groep 1: 1_F178; Groep 2: 5_R23647; Groep 3: 6_F141B en 7_F195; en Groep 4: 8_R23573, 9_R23581 en 10_R23577) wat geen verwantskap tot enige van die

huidige erkende tipe spesies van die *Chryseobacterium* genus getoon het nie wat aandui dat hierdie groepe verteenwoordigend van nuwe spesies kan wees.

Drie gekose stamme (8_R23573, 9_R23581 en 10_R23577) is aan ‘n polifasies taksonomiese studie onderwerp om hulle presiese taksonomiese identiteit te bepaal. Resultate van die mees algemene respiratoriese menakinone, vetsuur metielesters en DNA basis samestellings het die affiliasie met die *Chryseobacterium* genus bevestig. Toe die stamme aan DNA-DNA hibridisasie onderwerp is, het die stamme verwantskapwaardes van meer as 81% tussen die drie stamme getoon en minder as 57% ooreenkoms tussen die stamme en hulle twee naaste filogenetiese bure *C. shigense* (DSM 17126^T) en *C. luteum* (LMG23785^T). ‘n Nuwe spesies het te voorskyn gekom na vergelyking van die fenotipiese, chemotipiese en genotipiese resultate Die nuwe spesies is beskryf en die naam *Chryseobacterium carnipullorum* sp. nov. is voorgestel.

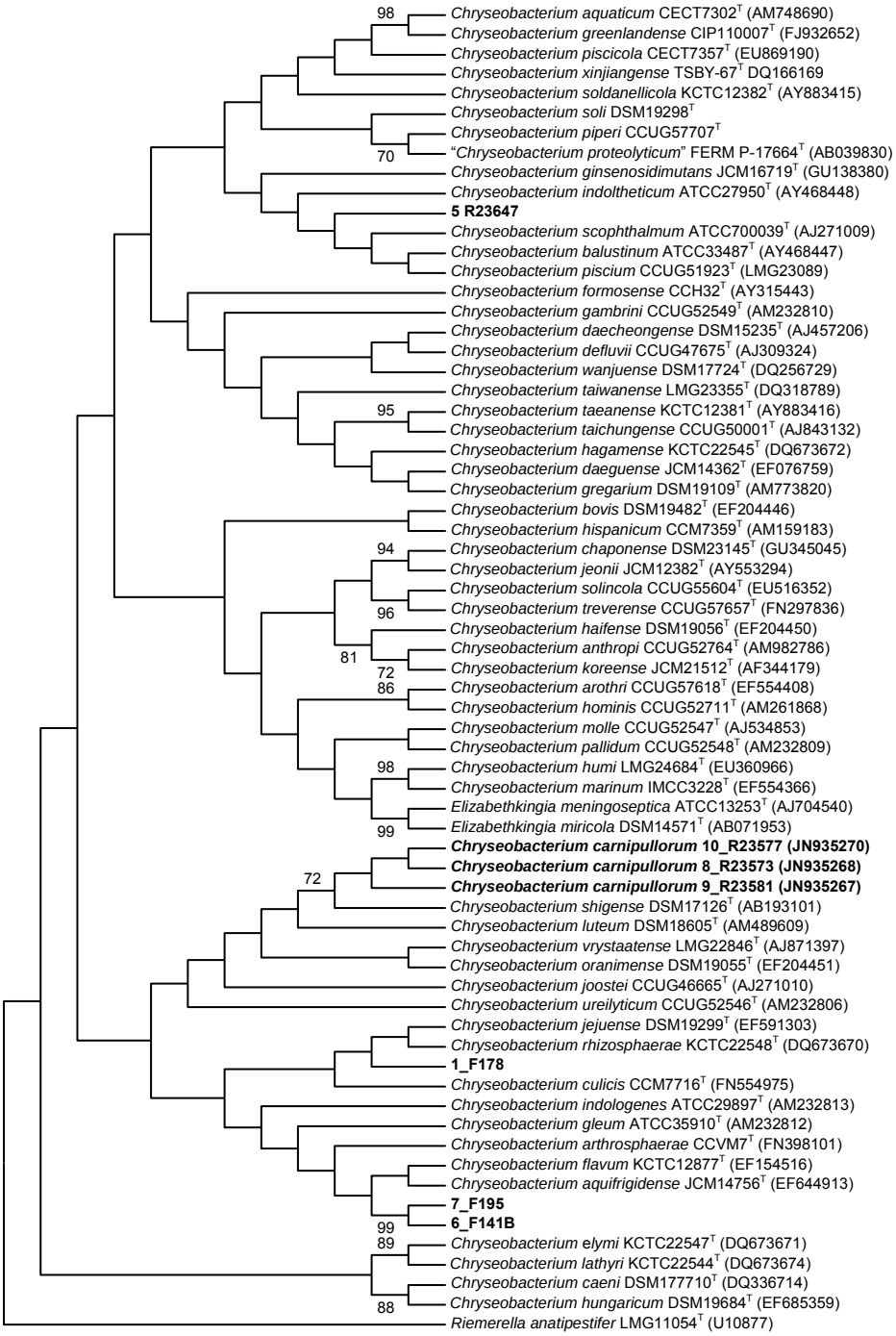
Analise met behulp van die BIOLOG “Phenotype MicroArray” (PM) stelsel het aangetoon dat *C. carnipullorum* die potensiaal het om voedsel te bederf hoofsaaklik deur die afbreek van koolhidrate, karboksielsure en aminosure waardeur metaboliete geproduseer word wat kan lei tot suur afsmake, galsterige gebreke, alkanisering, bitter afsmake en sulfiedbederf. Die organisme het ook die potensiaal getoon vir biotegnologiese toepassings in die voedsel- en veevoerbedrywe, tydens koffie-ekstraksie, in die olieontginning- en wasmiddel-bedrywe, in die vervaardiging van kunsmatige antigene en chemiese diagnostiese agente en vir die vrystelling van oligosakkariede en oligopeptiede in (ultra) oligotrofiese varswater omgewings.

Dit is bevind dat die nuwe spesies instaat was om ekstrasellulêre keratinases te produseer wat hoenderveerafval omvangryk bine 48 h kon afbreek. Dit het die potensiaal om ‘n oplossing te bied vir die opruimingsprobleem van groot hoeveelhede vere-afval wat in die pluimveebedryf ondervind word. Huidiglik word slegs ‘n klein persentasie vere-afval gestoom, chemies behandel en gemaal om as ‘n proteïenaanvulling vir veevoere te dien. Afbraak van vere met behulp van keratinolitiese organismes is ‘n ekonomiese- en omgewingsvriendelike alternatief. *Chryseobacterium carnipullorum* het ook die potensiaal vir toepassing in ander

biotegnologiese prosesse wat keratienhidolise betref. Gehidroliseerde vere kan omgeskakel word na bemestingstowwe, gomme, films en dit kan ook gebruik word as ‘n bron van seldsame aminosure soos serien, sisteïen en prolien.

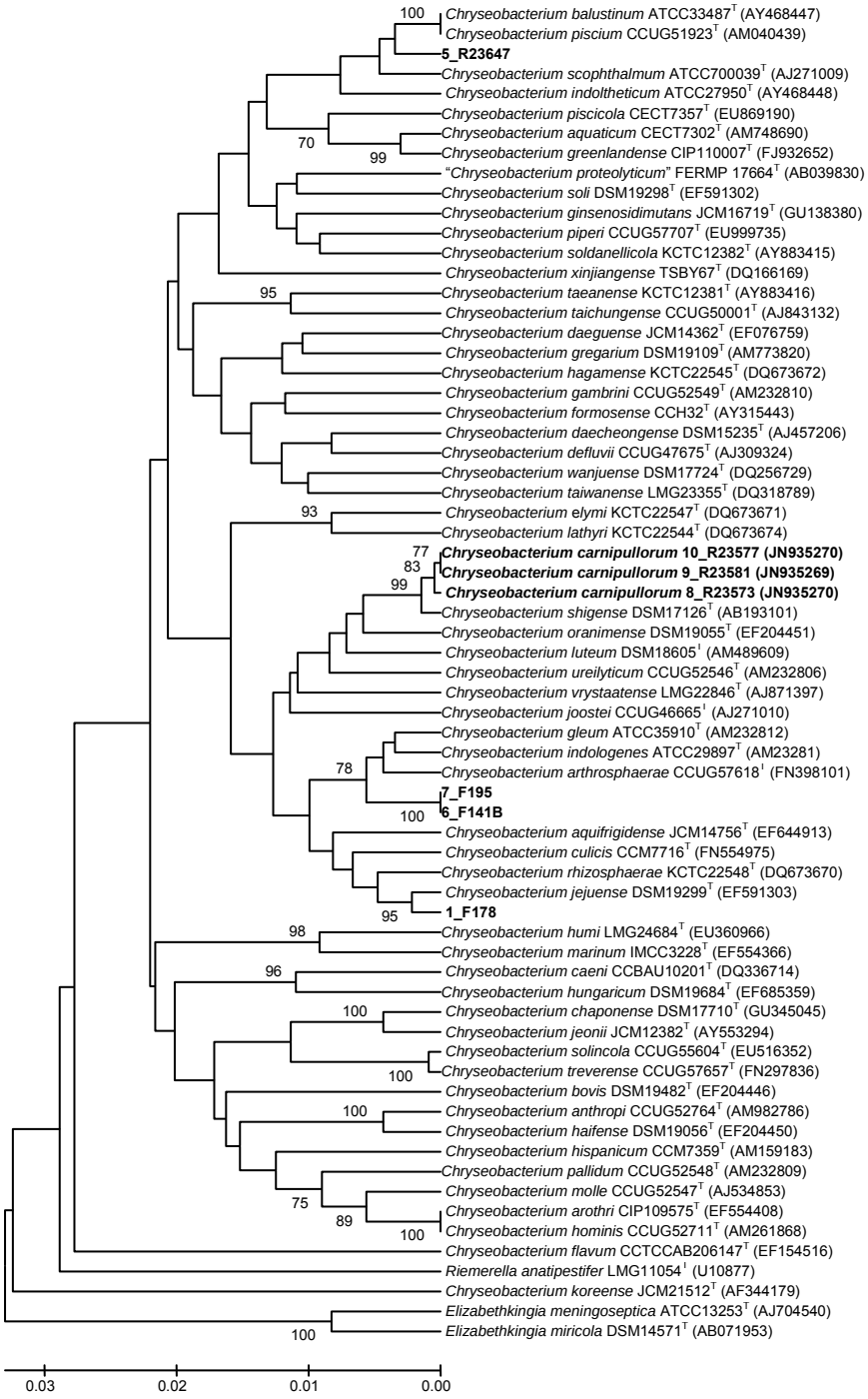
Sleutelwoorde: *Flavobacteriaceae, Chryseobacterium*, *C. carnipullorum*, taksonomie, keratinase.

Appendix I



Appendix I. Phylogenetic analysis of strains 1_F178, 5_R23647, 6_F141B, 7_F195, 8_R23573, 9_R23581 and 10_R23577, and all currently recognised *Chryseobaterium* type species based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses). Multiple alignments were performed. Clustering was determined using the maximum parsimony method (Eck and Dayhoff, 1966) in the MEGA version 4 software package (Tamura *et al*., 2007). Bootstrap values >70%, based on 1 000 replications, are given as percentages at the branching points.

Appendix II



Appendix II. Phylogenetic analysis of strains 1_F178, 5_R23647, 6_F141B, 7_F195, 8_R23573, 9_R23581 and 10_R23577, and all currently recognised *Chryseobaterium* type species based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses). Multiple alignments were performed and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the UPGMA method in the MEGA version 4 software package (Tamura *et al*., 2007). Bootstrap values >70%, based on 1 000 replications, are given as percentages at the branching points.