The Taxonomy and Spoilage Characteristics of *Flavobacteriaceae* Isolates from Food

by

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DECLARATION

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

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L.I. Tsôeu

May 2009

Dedicated to my parents, Samuel and Nomsa Tsôeu.

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LIST OF ABBREVIATIONS

C.	*Chryseobacterium*
E.	*Empedobacter*
W.	*Weeksella*
e.g.	for example
F	forward
Fig.	Figure
GN	Gram negative
h	hour(s)
UFSBCC	University of the Free State Bacterial Culture Collection
HCl	Hydrochloric Acid
MgCl	Magnesium Chloride
$mg.l^{-1}$	milligram per litre
min	minute(s)
N	Normality
sec	seconds
ml	millilitre
mM	millimolar
ppm	parts per million
µg	microgram
w/v	weight per volume
g	gram
R	reverse

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background to the study

The flavobacteria and its association with food spoilage, has long been recognized (Jooste and Britz, 1986). This group of bacteria is Gram-negative, yellow pigmented rods which can cause food spoilage because they are also proteolytic psychrotrophs. Food spoilage can be considered as any change which renders a product unacceptable for human consumption (Hayes, 1985).

The taxonomy of the flavobacteria has, however, undergone many changes in the past 10 to 12 years and has rapidly advanced in the past five years (Hugo and Jooste, in press). The family *Flavobacteriaceae* was first described in 1989 (Reichenbach) containing *Flavobacterium, Sphingobacterium* and *Weeksella* as genera (Holmes, 1992). With the emended description of *Flavobacteriaceae*, eight genera (*Flavobacterium, Bacteroides, Bergeyella, Chryseobacterium, Cytophaga, Empedobacter, Shingobacterium* and *Weeksella*) and the genera that would later become *Myroides* and *Tenacibaculum*, belonged to this family (Bernardet *et al.*, 1996). At the time of writing, however, this family consisted of 76 genera (Euzéby, 2008; Bernardet, in press).

It was found that the changes that took place in the family *Flavobacteriaceae* over the years were motivated by polyphasic studies. The increased accessibility of molecular sequencing techniques on one hand and the sharp increase in studies of the microbial ecology of various remote Antarctic and/or marine environments on the other, led to the discovery of not only new species, but also a large number of new genera that have been included in the family (Hugo and Jooste, in press).

It should be noted that many of the *Flavobacterium* species that were implicated and associated with the spoilage of food have been transferred to other genera in the family *Flavobacteriaceae* (Hugo and Jooste, 2003). Due to this reclassification and in some cases faulty classification of the flavobacteria, the information about the incidence and role of flavobacteria in food deterioration is challenging to obtain. Currently, only 10 of the 76 *Flavobacteriaceae* genera are associated with food, namely *Bergeyella, Chryseobacterium, Empedobacter, Flagellimonas, Flavobacterium, Myroides, Salegentibacter, Tenacibaculum, Vitellibacter* and *Weeksella* (Hugo and Jooste, in press).

The members of the family *Flavobacteriaceae* have been found in a wide range of habitats, but particularly in food they have been isolated from dairy products, meat and poultry, marine fish, molluscs, crustaceans and edible plants (Hugo and Jooste, in press). Jooste *et al*. (1985) were the first to isolate *Flavobacterium* CDC Group llb strains from milk and butter. Jooste *et al*. (1986) suggested that these *Flavobacterium* species caused putrefaction in salted butter by growing in cream prior to churning. In subsequent investigations (Hugo and Jooste, 1997; Hugo *et al*., 1999) a large group of the CDC Group llb milk isolates evaluated in the above mentioned studies were identified as *Chryseobacterium indologenes* and one isolate as *C. gleum*. Other groups emerging from this study mimicked the characteristics of some other flavobacteria and were grouped as *Chryseobacterium balustinum*-like, *Empedobacter*-like, *Weeksella*-like and a group which fell in an unidentified SDS-PAGE group in a later study by Hugo (1997).

A source of flavobacteria that was previously not investigated much but recently has come to the foreground, is vegetables (Liao and Fett, 2001; Young *et al.*, 2005; Beattie, 2006; Manani *et al*., 2006). The flavobacteria in these studies have been isolated from all over the vegetable plant and also from soil surrounding the vegetables. In many of these studies, the identification of the

flavobacteria was only done by rapid identification techniques with no confirmation of identity by genomic techniques. It is, therefore, still unknown which genera of the *Flavobacteriaceae* mainly occur on vegetables.

1.2 Purpose, hypotheses and objectives of the study

1.2.1 Purpose

i). To give an overview of the *Flavobacteriaceae* family in terms of taxonomy and habitat, and then to focus on the food-associated *Flavobacteriaceae* in terms of sources of isolation and spoilage characteristics.

ii). To subject a range of newly and previously isolated food and environmental flavobacteria to the newest taxonomic techniques for more correct classification of isolates.

iii). To determine the possible spoilage characteristics of the isolates in the resulting taxonomic groups that are relevant to the different food commodities.

1.2.2 Hypotheses

i). The variety of flavobacteria (yellow pigmented Gram-negative bacteria) on vegetables and surrounding soil is not known. Which genera of the *Flavobacteriaceae* family do occur on vegetables and surrounding soil and which vegetables are sources?

ii). The re-examination of preserved flavobacterial strains previously isolated from dairy products using newer phenotypic and molecular methods is fundamental to place these strains in their rightful place in the current taxonomy of the family *Flavobacteriaceae*.

iii). Since the way in which flavobacteria spoil food has not been extensively studied, this research will expose flavobacteria to different carbon sources, most likely found in many foods, to better understand their spoilage characteristics.

1.2.3 Objectives

i). Isolate yellow pigmented colonies from a wide range of vegetables and the surrounding soil associated with each vegetable where soil was available.

ii). Identify the yellow pigmented colonies from the vegetables using phenotypic characteristics, the BIOLOG system and 16S rRNA sequencing.

iii). Determine possible spoilage characteristics of the vegetable isolates by using the BIOLOG system.

iv). Re-evaluate and identify the dairy isolates from the study of Jooste (1985) and Hugo (1997) which grouped as *Chryseobacterium balustinum*-like, *Empedobacter*-like, *Weeksella*-like and unidentified SDS-PAGE group by using phenotypic characteristics, the BIOLOG system and 16S rRNA sequencing.

v). Determine the possible spoilage characteristics of the identified dairy isolates by using the BIOLOG system. The main purpose of this study was to subject a range of newly and previously isolated food and environmental flavobacteria to the newest taxonomic techniques for more correct classification of these isolates and to determine their possible spoilage characteristics in sources where isolated.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The family *Flavobacteriaceae* was first suggested by Jooste in 1985. The name of the family was only validated in 1992 by Reichenbach and its description was subsequently emended (Bernardet *et al*., 1996; 2002). Many new organisms have been allocated to the family over the past few years (Bernardet and Nakagawa, 2006). Members of the family *Flavobacteriaceae* are widely distributed in diverse habitats (Bernardet *et al*., 2002).

One of these habitats includes marine surroundings where flavobacteria are often associated with living and dead phytoplankton. They also colonize living algae, absorbing nutrient exudates produced during photosynthesis (Bowman and Nichols, 2002). Those living in soil and water frequently synthesize cellulose, pectin, xylan and chitin-degrading enzymes that decompose dead plants, fungi and insects (Reichenbach, 1989; Johansen and Binnerup, 2002). Members of the *Flavobacteriaceae* family differ widely in their enzymatic abilities. The array of enzymes produced by flavobacteria however depends on the major biopolymers available in their habitats (Kirchman, 2002).

Members of the family *Flavobacteriaceae* have also been isolated from a variety of food products such as milk and dairy products, poultry and poultry products, meat and meat products and vegetables during commercial processing (McMeekin *et al*., 1971; Hayes, 1977; Holmes *et al*., 1984a). Jooste and Britz (1986) demonstrated that some flavobacteria were highly proteolytic and the possible heat-resistance of these proteinases, when introduced into raw milk from poorly sanitized equipment, can have an adverse effect on milk and dairy products after processing. In a study to determine spoilage in pelagic fish, Engelbrecht *et al*. (1996) evaluated the

spoilage potential of chryseobacteria in fish muscle extract. Several *Chryseobacterium* strains were found to produce pungent and stale odours due to their proteolytic activity.

Members of the family *Flavobacteriaceae* have been found to have both positive and negative impacts on the environment. The positive impacts include synthesis of a number of enzymes that are potentially useful in industry or medicine or that contribute to the turnover of organic matter in soil, water and sewerage plants. Negative impacts include spoilage defects in food and infections in humans and animals (Bernardet *et al*., 2006).

The aims of this literature review, consequently, were first to give an overview of the *Flavobacteriaceae* family in terms of taxonomy and habitat. In the second place the literature review will focus on food-associated *Flavobacteriaceae* in terms of source and spoilage characteristics.

2.2 Taxonomy

The genus *Flavobacterium* was destined to suffer the same fate as many other earlier established genera, since its original description relied on parameters which are now considered to have little taxonomic significance. This genus comprised of a collection of predominantly yellow-pigmented bacteria that were, according to modern genotypic standards, not at all closely related (Vandamme *et al*., 1994a).

2.2.1 Background

The name *Flavobacterium* was proposed in 1923 for a genus of rod-shaped, non-endosporeforming, chemo-organotrophic bacteria (Bergey *et al*., 1923). This genus was included in the tribe *Chromobacteriaceae* of the family *Bacteriaceae*. Apart from the genus *Flavobacterium*, the tribe *Chromobactericeae* also included the genera *Serratia, Chromobacterium* and *Pseudomonas*. These genera represented the yellow, red, violet and green pigmented bacteria respectively. Any new species of yellow pigmented

bacteria was thereafter placed in the genus *Flavobacterium*. Taxonomic heterogeneity and general uncertainty have characterized *Flavobacterium* from its inception and its history has been a record of proposals aimed at achieving credibility for the genus (Bernardet *et al.*, 2006).

Through successive emendations, the genus *Flavobacterium* became restricted to non-motile and non-gliding species and consequently achieved what could be considered reasonable homogeneity in the 1984 edition of *Bergey's Manual of Systematic Bacteriology* (Holmes, 1984a).

The family *Flavobacteriaceae* was first suggested by Jooste in 1985. The name of the family was validated in 1992 by Reichenbach and its description was subsequently emended (Bernardet *et al*., 1996; 2002). Except for the genus *Flavobacterium*, many new genera have been allocated to the family over the past few years (Bernardet and Nakagawa, 2006). The reason for this can be ascribed to polyphasic taxonomic approaches (Vandamme *et al*., 1996a) supported by 16S rRNA gene sequence-based phylogeny. At present the genus *Flavobacterium* (Bergey *et al*., 1923, emend. Bernardet *et al*., 1996) is the type genus of the family *Flavobacteriaceae.* At the date of writing, *Flavobacteriaceae* consisted of 76 recognized genera (Euzéby, 2008). These genera, each with its type species and source of isolation, are presented in Table 2.1. Of the 76 genera, only *Bergeyella, Chryseobacterium, Empedobacter, Flagellimonas, Flavobacterium, Myroides, Salegentibacter, Tenacibaculum, Vitellibacter* and *Weeksella* have been found to be associated with food (Hugo and Jooste, in press).

2.2.2 Characteristics

The latest emended description of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002) is as follows: cells are short to moderately long rods with parallel or slightly irregular sides and rounded or slightly tapered ends. They are usually 0.3 to 0.6 µm wide and 1 to 10 µm long though members of some species may form filamentous flexible cells (e.g. *Flavobacterium* and

Table 2.1. **Currently recognized genera and type species classified in the family** *Flavobacteriaceae* **(Euzéby, 2008).**

Genus	Type species	Source	Reference(s)
Aequorivita	*Aequorivita antarivita*	Under-ice sea water	Bowman & Nichols, 2002
Actibacter	*Actibacter sediminis*	Tidal flat sediment	Kim *et al.*, 2008a
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	Ivano *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 & 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 & 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 brevis*	Clinical- human	Holmes *et al*., 1978; Holmes *et al*., 1984a; Bernadet *et al*., 1996
Epilithonimonas	*Epilithonimonas tanax*	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
Flavobacterium	*Flavobacterium aquatile*	Fresh and salt water, fish, soil	Holmes *et al*., 1984a; Bernardet *et al*., 1996
Flaviramulus	*Flaviramulus basaltis*	Seafloor basalt	Einen and Øvreas, 2006
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
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
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 & Nichols, 2005
Leeuwenhoekiella	*Leeuwenhoekiella blandensis*	Algal blooms	Pinhassi *et al*., 2006
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 rhinotrachealle*	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
Polaribacter	*Polaribacter filamentus*	Fresh and salt water	Gosink *et al*., 1998
Psychroflexus	*Psychroflexus torquis*	Salt water	Bowman *et al*., 1998
Psychroserpens	*Psychroserpens burtonenensis*	Salt water	Bowman *et al*., 1997a
Riemerella	*Riemerella anatipesticer*	Clinical & poultry	Segers *et al*., 1993
Robiginitalea	*Robiginitalea biformata*	Marine habitat	Cho & Giovannoni, 2004
Salegentibacter	*Salegentibacter salegens*	Organic water	Dobson *et al*., 1993; McCammon & 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 – penquin 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 wynnwilliamsii*	Quartz stone cyanobacterial biofilm	Bowman & Nichols, 2005
Subsaximicrobium	*Subsaximicrobium wynnwilliamsii*	Antarctic maritime habitats	Bowman & Nichols, 2005
Tamlana	*Tamlana crocina*	Beach sediment	Lee, 2007
Tenacibaculum	*Tenacibaculum maritimum*	Marine environment	Wakabayashi *et al*., 1986; Bernadet

			& 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 thalasscola*	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

Tenacibaculum) or coiled and helical cells (*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*). The organisms are Gram-negative and non-sporeforming. Gas vesicles are produced in members of some *Polaribacter* species. Flagella are usually absent. In the case of *Polaribacter irgensii*, the only strain available is flagellated, but motility has not been observed in wet mounts. Genera are usually non-motile (*Bergeyella*, *Chryseobacterium*, *Coenonia*, *Empedobacter*, *Myroides, Ornithobacterium*, *Polaribacter*, *Psychroserpens*, *Riemerella*, *Salegentibacter, 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*, *Gelidibacter*, *Myroides, Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Weeksella*, and *Zobellia* strains) or microaerobic to anaerobic (*Capnocytophaga*, *Coenonia*, *Ornithobacterium*, and *Riemerella* strains). The optimum temperature is usually in the range of 25 °C to 35 °C, but members of some species or genera are psychrophilic or psychrotrophic (*Flavobacterium psychrophilum* and the Antarctic *Flavobacterium* species, as well as *Gelidibacter*, *Polaribacter*, *Psychroflexus, Psychroserpens*, and *Salegentibacter* strains). Members of some taxa are halophilic to varying degrees (*Cellulophaga*, *Gelidibacter*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum* and *Zobellia* strains).

Colonies may be 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. The organisms are chemo-organotrophic. Intracellular granules of poly-β-hydroxybutyrate are absent. Sphingophospholipids are absent. Homospermidine is the major polyamine although 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 G+C mol%.

The members of the *Flavobacteriaceae* family are mostly saprophytic in their terrestrial and aquatic habitats. Certain members are commonly isolated from diseased humans or animals and 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).

The above mentioned characteristics are applicable to all the genera in the *Flavobacteriaceae* family. Since this study will focus on food associated isolates, the differential characteristics of the food *Flavobacteriaceae* members are presented in Table 2.2.

Table 2.2 Differential characteristics for the food associated members of the *Flavobacteriaceae* **family.** Data from Hugo and Jooste (in press). +, positive reaction; -, negative reaction; (+), weak positive or delayed reaction; C, carotenoid; F, flexirubin; ND, no data available; O, orange; PYR, pyrrolidonyl arylamidase activity; V, variable reaction; v, varies between references; Y, yellow; (Y), light yellow.

Characteristic	*Bergeyella*	*Chryseobacterium*	*Empedobacter*	*Flagellimonas*	*Flavobacterium*	*Myroides*	*Salegentibacter*	*Tenacibaculum*	*Vitellibacter*	*Weeksella*
Pigment	-	+ F	(+)	+ C	+F and/or C	+ F	+ C	+ C	+ F	-
Colony pigment	Tan to yellow	Y	(Y)	O	Y	(Y)	Y	Y	Y-O	Tan to brown
Motility	-	-	-	+	-	-	-	ND	-	-
Gliding motility	-	-	-	-	V	-	-	+	-	-
Sea water requirement	-	-	-	+	-	-	-	V	-	-
Capnophilic metabolism	-	-	-	-	-	-	-	-	-	-
Growth at (°C):										
25	+	+	+	+	V	+	+	+	+	+
37	+	V	+[a]	-	-	+	-	V	+	-
42	-[b]	V	-	-	-	-	-	-	+	-

Table 2.2 Continued.

Characteristic	*Bergeyella*	*Chryseobacterium*	*Empedobacter*	*Flagellimonas*	*Flavobacterium*	*Myroides*	*Salegentibacter*	*Tenacibaculum*	*Vitellibacter*	*Weeksella*
Growth on agar:										
MacConkey	-	V	+	ND	ND	+	ND	ND	ND	+
β-hydroxybutyrate	-	V	+	ND	ND	+	ND	ND	ND	+
Acid production from:										
Glucose	-	+[c]	+[a]	-	V	-	V	ND	-	-
Sucrose	-	v	-	ND	V	-	V	ND	-	-
Production of:										
DNase	-	+	+	ND	V	+	+	+	+	-
Urease	+	V	V	-	V	+	-	ND	-	-
Oxidase	+	+	+	-	V	+	+	+	+	+
Catalase	+	+	+	ND	+/(+)	+	+	+	+	+
Indole	+	+[c]	+	ND	-	-	ND	ND	-	+
β-Galactosidase	-	V	-	+	V	-	+	ND	-	-
H_2S	-	-	-	-	V	ND	+	-	-	-

Table 2.2 Continued.

Characteristic	*Bergeyella*	*Chryseobacterium*	*Empedobacter*	*Flagellimonas*	*Flavobacterium*	*Myroides*	*Salegentibacter*	*Tenacibaculum*	*Vitellibacter*	*Weeksella*
Nitrate reduction	-	V	-	-	V	-	+	V	-	-
Carbohydrate utilization	-	+	V	-	V	-	+	ND	+	-
Degradation of:										
Agar	-	-	-	-	V	-	-	-	-	-
Starch	-	V	v	-	V	-	+	V	-	-
Esculin	-	+	-	ND	V	-	+	-	ND	-
Gelatin	+	+	+	-	V	+	+	+	+	+
PYR activity	-	+	ND	ND	ND	+	ND	ND	ND	+
Resistance to Pen G	-	+[d]	+	ND	V	ND	ND	-[e]	ND	-

[a] Positive for most strains

[b] Negative for most strains

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

[d] Not determined for '*C. proteolyticum*'

[e] Negative for *T. maritimum*, not determined for other *Tenacibaculum* species

2.2.3 Phylogeny

Historically, bacteria belonging to the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB) have been poorly investigated in terms of their phylogeny. During the last decade, many novel taxa belonging to the phylum CFB have been described, and some bacterial species that previously had unclear taxonomic positions within this phylum have been reclassified due to the use of a polyphasic taxonomic approach (Vandamme *et al*., 1996a). The current view of phylogenetic relationships of the family *Flavobacteriaceae* with other taxa in the CFB phylum and among taxa in the family mostly results from extensive 16S rRNA/rDNA sequence analyses and DNA-rRNA hybridization experiments performed over the past decade or more (Gherna and Woese, 1992; Nakagawa and Yamasato, 1993; Bernardet *et al*., 1996; Bowman *et al*., 1998; Suzuki *et al*., 2001; Nakagawa *et al.*, 2002).

Procedures to determine the almost complete base pairs 16S rRNA sequence of approximately 1400 base pairs as well as efficient methods of alignment, treeing algorithms and statistical analyses, are now readily available. This has made it possible to resolve phylogenetic relationships much more accurately and reliably (Bernadet *et al*., 2002). The phylogenetic relationships among the *Flavobacteriaceae* representatives based upon 16S rRNA sequences are presented in Fig. 2.1.

2.3 Natural habitats of genera in the family *Flavobacteriaceae*

Members of the family *Flavobacteriaceae* are found in a variety of environments. Essential populations of CFB (*Cytophaga-Flavobacterium-Bacteroides*) are found frequently in freshwater environments (Manz *et al*., 1999; Brummer *et al*., 2000; Kirchman, 2002), marine environments (Cottrell and Kirchman, 2000; Eilers *et al*., 2000; Kirchman, 2002), polar regions (Bowman *et al*., 1997b; Ravenschlag & Dworkin, 2001) and industrial environments (Whiteley and Bailey, 2000). They habitually occur in biofilms (Kirchman, 2002) and seem to play a role in bio-geochemical cycles, using their diverse enzymes to degrade a variety of complex organic substrates in

natural habitats (Reichenbach, 1989; Kirchman, 2002; Johansen and Binnerup, 2002; Bernardet and Bowman, 2006). The array of enzymes produced by flavobacteria understandably depends on the major biopolymers available in their habitats (Kirchman, 2002).

Flavobacteria have also been isolated from the habitats such as soil, food and dairy products, eggs, diseased dogs and cats, diseased amphibians and reptiles, digestive tract of insects and diseased plants, diseased freshwater and marine fish (Bernardet and Nakagawa, 2006). Flavobacteria have also been isolated from clinical specimens in hospital surroundings and from devices such as blood, urine, infected wounds and faeces of patients (Holmes, 1992).

2.4 The history of flavobacterial research at the University of the Free State

The interest in flavobacteria at the University of the Free State started in the 1980's, when Prof. P.J. Jooste discovered during his Ph.D. study (Jooste, 1985) that these bacteria occur in milk and butter. It was also in this study that the family name, *Flavobacteriaceae*, was proposed. This family name was then mentioned by Reichenbach (1989) in the first edition of *Bergey's Manual of Systematic Bacteriology* but not formally described. The family was validated in 1992 (Reichenbach, 1992) and its description published in 1996 (Bernardet *et al*., 1996). The latest emendation of the family *Flavobacteriaceae* was by Bernardet *et al*. (2002).

After the emendation of the family *Flavobacteriaceae,* successive studies were carried out, at the University of the Free State, on the presence of these bacteria in food. The genera *Bergeyella* and *Weeksella* were previously known as CDC groups IIf and llj respectively. Strains resembling these organisms were isolated from meat and dairy sources (Jooste *et al*., 1985). Later *Bergeyella* and *Weeksella*-like organisms were also isolated from food sources such as raw beef, pork, chicken and lamb portions. However, these organisms did not warrant inclusion in the genera *Bergeyella* and

Weeksella (Botha *et al*., 1989; Botha *et al*., 1998a and b) because of their susceptibility to antimicrobial agents, non-saccharolytic nature, inability to produce yellow pigment and in being strict parasites rather than free-living organisms.

Strains of the genus *Empedobacter,* a genus that was formerly included in the genus *Flavobacterium,* were isolated from South Atlantic fish species at the processing site in the Western Cape region of South Africa and were considered potential active spoilers of fish (Engelbrecht *et al*., 1996).

The so called CDC Group llb organisms, of which most isolates are now regarded to be members of the *Chryseobacterium* genus, have been the focus of a few studies at the University of the Free State (Hugo, 1997; Hugo *et al*., 1999). These studies included the purification and characterization of a metalloprotease from *C. indologenes* (Venter *et al.*, 1999), differentiation and taxonomy of food strains of *Chryseobacterium* and *Empedobacter* (Hugo and Jooste, 1997; Hugo *et al.*, 1999), description of new species (*C. joostei* - Hugo *et al.*, 2003; *C. vrystaatense* - de Beer *et al.*, 2005; *C. piscium* - de Beer *et al.*, 2006), description of potential spoilage characteristics of flavobacteria (Mielmann, 2006) and potential pathogenic characteristics of these organisms (van Wyk, 2008).

Reviews and chapters in books from the University of the Free State which contributed and still contribute to the knowledge on the *Flavobacteriaceae*, included reviews on the taxonomy, ecology and cultivation of *Flavobacteriaceae* genera (Jooste and Hugo, 1999); chapters on cultivation media for the food *Flavobacteriaceae* (Hugo and Jooste, 2003; in press). Chapters were contributed to *The Prokaryotes* on *Bergeyella* and *Weeksella* (Hugo *et al.*, 2004a; 2006a), on *Empedobacter* and *Myroides* (Hugo *et al.*, 2004b; 2006b) and on *Chryseobacterium* and *Elizabethkingia* (Bernardet *et al.*, 2006). A chapter on *Chryseobacterium* will also appear in the latest edition of *Bergey's Manual* (Bernardet *et al.*, in press).

2.5 The food-associated *Flavobacteriaceae* and their food spoilage characteristics

As mentioned above, only 10 of the 76 currently described *Flavobacteriaceae* genera are associated with food. These genera occur in diverse food habitats which will be discussed in the subsections that follow. In this literature review attention will be focused especially on *Bergeyella*, *Chryseobacterium, Empedobacter, Flavobacterium, Myroides* and *Weeksella* from food sources, since these genera have been isolated from food on a more regular basis than the four other food-associated *Flavobacteriaceae* genera.

2.5.1 Milk and milk products

Members of the family *Flavobacteriaceae* have been long known for their psychrotrophic characteristics and their potential to decompose milk and dairy products (Gilmour and Rowe, 1981; Cousin, 1982). In the case of milk production, raw milk entering the dairy plant contains Gram-negative psychrotrophic bacteria with the capacity to grow and multiply in refrigerated milk. Post-pasteurization recontamination frequently takes place during the filling procedure (Schröder, 1984; Eneroth *et al*., 2005; Walker, 2007) which is an open process and allows the milk to come in contact with the surrounding air. Condensed water on the machinery may find its way into the milk and insufficiently cleaned surfaces in the filling machine can come into contact with the inside of the package or with the milk (Griffiths and Phillips, 1990).

Milk contains ample nutrient ingredients that will ensure not only survival, but also growth of microorganisms. Such growth of proteolytic flavobacteria (Cousin, 1982; Jooste, 1985) can result in the production of pasteurisation resistant extracellular enzymes. This in turn can contribute to the proteolytic spoilage of milk and dairy products. Heat-stable proteases produced by proteolytic bacteria in the raw milk, break down casein and increase low-molecular weight nitrogen compounds that can lead to defects and spoilage in pasteurised products (Fairbain and Law, 1986). When

growing in milk, Gram-negative bacteria can produce sufficient proteinases to hydrolyse all of the available casein into soluble peptides. The major defect caused by the action of these enzymes is the development of unclean or bitter flavours in and gelation of long-life heat treated milk (Meer *et al*., 1991).

Lipase activity has been reported for most psychrotrophs isolated from milk and milk products. Most microbial lipases have been found to be highly resistant to heat. Lipase activity in milk leads to the preferential release of medium and short-chain fatty acids from triglycerides. Hydrolysis and release of as little as 1-2% short chain triglycerides can result in rancid off-flavours (Roussis *et al*., 1999).

A number of genera in the family *Flavobacteriaceae* have been isolated from dairy products. CDC Group llf-like flavobacteria (now better known as *Weeksella*) were found to grow in contaminated pasteurized cream prior to churning, resulting in putrid defects in the churned butter due to their proteolytic activity (Jooste, 1985). In other taxonomic studies, Botha *et al*. (1989) also showed that *Weeksella*- and *Bergeyella*-like strains could be isolated from dairy sources.

A study by Jooste (1985) revealed that a large number of raw milk isolates also belonged to CDC Group IIb flavobacteria. Later studies on these isolates (Hugo, 1997; Hugo *et al.*, 1999) more correctly placed these isolates in the *Chryseobacterium* genus with a large number of the isolates being found to belong to the *C. indologenes* species. In further studies, a *C. indologenes* isolate (Ix9a) from the latter studies proved to be a potential milk spoilage agent by producing very heat stable metallo-proteases which have a great affinity for the casein proteins in milk (Venter *et al.*, 1999).

Other studies on milk and milk products also revealed the presence of novel *Chryseobacterium* species: three strains of *C. bovis* (Hantsis-Zacharov *et al.*, 2008) and one strain of *C. haifense* (Hantsis-Zacharov and Halpern, 2007) were isolated from raw cow's milk during a study of the diversity of psychrotolerant bacteria in raw milk in Israel. In South Africa *C. joostei* (Hugo

et al., 2003) and *C. gleum* were also isolated from raw milk (Jooste *et al.*, 1985; Welthagen and Jooste, 1992; Hugo *et al.*, 1999). In Japan, *C. shigense* was isolated from a lactic acid beverage (Shimomura *et al.*, 2005) and *C. indologenes* was isolated from goat milk in France (Callon *et al.*, 2007). The spoilage characteristics of these species have, however, not been investigated.

2.5.2 Meat and meat products

Flavobacteria in meat and meat products were first reported by McMeekin *et al*. (1971; 1972) who isolated these organisms from processed meats. In 1977, Hayes isolated and divided a large number of flavobacterial isolates and related Gram-negative, yellow pigmented rods into nine phena, the first five phena being found to belong to the then genus *Flavobacterium*. In a study of these organisms by Owen and Holmes (1980), the conclusion was drawn that Hayes' phenon 1 corresponded closely to CDC Group llb flavobacteria, of which some members were later included in the species *C. indologenes* and *C. gleum*. Flavobacteria have also frequently been isolated from meat and poultry by other workers (García-López *et al*., 1998; Olofsson *et al.*, 2007), although they were not identified to a species level.

The high protein content of meat results in its predictable, perishable nature and spoilage takes place even at refrigeration temperatures. Spoilage of raw meat results in undesirable off-odours and flavours due to metabolic end products, possible slime production, and discolouration of specific surfaces of the product (Labadie, 1999). Metabolites possibly involved in spoilage defects in meat and produced by flavobacteria, include alcohols such as methanol and ethanol, sulphur compounds such as dimethylsulphide, methylmercaptan and methanethiol, ketones, aldehydes, esters and amines from amino acid catabolism (Banwart, 1989). These products result in off- or mal-odours described as fishy, foul, sulphuric and ammonia-like (Nychas and Drosinos, 1999).

2.5.3 Poultry

The nutrient composition of poultry muscle is similar to that of red meats and thus the mechanism of microbial spoilage is similar (Bryan, 1980). Daud *et al*. (1979) studied the microflora of chicken meat at 2°C and found this to include species of *Micrococcus, Flavobacterium, Cytophaga, Acinetobacter, Moraxella* and *Pseudomonas*, as well as enterobacteria. Recently, *Chryseobacterium vrystaatense* was isolated from the skin of raw chickens in a broiler processing plant (de Beer *et al*., 2005). Geornaras *et al*. (1996) and Ellerbroek (1997), also conducted studies on poultry and on the air in poultry establishments and discovered the presence of *Flavobacterium*. Poultry skin and muscle provide excellent growth substrates for spoilage microorganisms, but spoilage is generally restricted to the outer surfaces of the skin and cuts and has been characterized by off-odours, sliminess, and various types of discolouration.

2.5.4 Fish

Sea food is harvested from sea water. Microorganisms in the water contaminate the surface, gills, and intestinal tract of fish (Leclerc and Moreau, 2002). Therefore, the isolation of the genus *Flavobacterium* was not surprising since they have long been known to belong to the normal bacterial populations of freshwater fish and fish eggs (Bernardet and Bowman, 2006).

Gram and Huss (1996), found that trimethylamine oxide reduction to trimethylamine catalysed by trimethylamine-*N*-oxide reductase readily occurs in fish, resulting in strong mal-odours. However, spoilage of fish has also been accompanied with off-odours associated with the breakdown of sulphur-containing amino acids such as cysteine and methionine. These odours are attributed to volatile products such as hydrogen sulphide, methyl mercaptan and dimethyl sulphide (Engelbrecht *et al*., 1996; Huis in't Veld, 1996). The catabolites generally produced by spoilage organisms in fish include ammonia, amines and sulphides (Gram and Dalgaard, 2002). Excessive

bacterial growth on the fish surfaces can result in the formation of visible, pigmented colonies (Walker and Stringer, 1990).

Chryseobacterium balustinum was the first organism in the *Flavobacteriaceae* family to be isolated from fish scales of freshly caught halibut in the Pacific Ocean (Harrison, 1929). González *et al*. (2000) also isolated this organism from the skin and muscle of wild and farmed freshwater fish that had been stored for more than three days in melting ice. Proteolytic activity and hydrogen sulphide production has also been ascribed to *C. balustinum*, *C. gleum* and *C. indologenes* isolated from Cape marine fish in South Africa (Engelbrecht *et al*., 1996). Four strains of *C. piscium* were isolated from fish caught in the South Atlantic Ocean near South Africa (de Beer *et al.,* 2006).

Empedobacter and *Myroides* strains were isolated from Cape hake and other South Atlantic fish species and were considered potentially active spoilers of the fish in terms of off-odour production (Engelbrecht *et al.*, 1996). These two organisms were also isolated from freshwater fish skin, the water of the sampling site, and during chill storage by Gonzalez *et al*. (2000) but were not considered important contributors to the spoilage of these fish by the latter workers.

Chryseobacteria have also been found to be pathogenic in fish. *Chryseobacterium scophthalmum* (Mudarris *et al.*, 1994) and *C. joostei* for example were isolated from diseased fish and *C. joostei* is regarded as an emergent pathogen in various fish species (Bernardet *et al.*, 2005). *Chryseobacterium arothri* was isolated from the kidneys of a pufferfish (*Arothron hispidus*) in the warm tropical waters around the Hawaiian Islands. It is not known whether this species is pathogenic to the fish or whether it could have been involved in *post mortem* growth in the fish (Campbell *et al.*, 2008).

Most “authentic” *Flavobacterium* species today (e.g. *F. branchiophilum, F. columnare, F. johnsonia, F. psychrophilum)* are regarded as fish pathogens

rather than spoilers (Bernardet and Bowman, 2006). In a study by Smith *et al.* (1984), however, shrimp spoilage, due to indole production, was attributed to "*Flavobacterium"* species which made up 52.4% of the total microflora found on the spoiled shrimps. High percentages (43%) of flavobacteria also formed part of the bacterial population on Indian white shrimp (Jeyasekaran *et al.*, 2006).

2.5.5 Vegetables

Vegetables are expected to contain relatively high numbers of microorganisms at harvest because of their contact with the soil during growth, but some soil organisms, however, do not attach to plants. Those that persist on plant products do so by virtue of a capacity to adhere to plant surfaces so that they are not easily rinsed off and because they are able to fulfil their nutritional requirements. Not all microorganisms are, however, capable of proliferating on vegetables. The numbers of microorganisms on fresh vegetables can vary from location to location and reflect the growth environment and the handling and storage conditions after harvest (Tournas, 2005).

Food products of vegetable origin present a special case due to the nutrient composition of these products. The high pH will allow a range of Gram-negative bacteria to grow, but spoilage is specifically caused by organisms capable of degrading the vegetable polymer, pectin (Liao, 1989; Liao *et al*., 1997). Plant material consists of water, carbohydrates and related compounds (starch and cellulose), proteins, peptides, amino acids, lipids, vitamins and minerals (Jay, 1996).

Liao and Fett (2001) isolated *Flavobacterium* species from green bell peppers, Romaine lettuce and baby carrots. Their role in these vegetables was, however, not explained. Banwart (1989) discovered that some *Flavobacterium* species produced discolourations on thawed and fresh vegetables. Manani *et al*. (2006) examined the microbiology of minimally processed frozen and pre-packed potato chips, peas, corn and a variety of

combined vegetables from supermarkets in Gaborone, Botswana. Different types of bacteria were isolated from the study. Among the Gram-negative isolates believed to form part of the spoilage microflora, *Flavobacterium* was reported to contribute to the spoilage of vegetable products destined to be frozen. Jay *et al.* (2003) reported *Flavobacterium* to occur commonly in fresh vegetables and to be involved in the low-temperature spoilage of these products.

Some *Chryseobacterium* species have been isolated from edible plants where they play a beneficial role by exhibiting antagonistic traits against plant pathogens. These chryseobacteria include *C. balustinum* from peppers and tomatoes (Domenech *et al*., 2006) and also from potatoes (Krechel *et al.*, 2002). *Chryseobacterium soldanellicola* and *C. taeanense,* that also show beneficial tendencies, were isolated from the roots of sand dune plants in Korea (Park *et al*., 2006). *Chryseobacterium indologenes* and *C. balustinum* have also been isolated from sugar beet leaves (Beattie, 2006), while *C. formosense* was isolated from the rhizosphere of garden lettuce (Young *et al.*, 2005). The role of the latter species, however, was not clear. *Myroides odoratus* has been isolated from the geocarposphere of peanuts (Chourasia, 1995).

Plant spoilage microorganisms excrete lytic enzymes, which break down plant components. Pectinolytic enzymes such as polygalaturonases, pectin esterase and pectate transeliminases break down pectin by splitting glucosidic bonds. This process gives rise to a soft, mushy consistency, sometimes a bad odour and a water soaked appearance (Banwart, 1989).

2.5.6 Soil

Soil is the natural habitat of many microorganisms. The types and numbers of microorganisms vary with the type of soil and with environmental conditions (Fent, 1996). *Chryseobacterium indologenes* strains, recently recognized from their 16S rRNA gene sequences, were isolated from soil samples in Indonesia and Spain and were shown to degrade various toxic

compounds present in the soil (Lopez *et al*., 2004). The recent description of another novel soil species of *Chryseobacterium*, namely *C. proteolyticum*, was based on two strains isolated from the soil of a rice field and from the bank of a brook in Japan (Yamaguchi and Yokoe, 2000).

Two other new species of *Chryseobacterium* (*C. formosense* and *C. taichungense*) were isolated from the rhizosphere of garden lettuce and from a sample of contaminated soil, respectively (Young *et al*., 2005; Shen *et al*., 2005). The rhizosphere bacteria benefit from the diffusion of a wide variety of soluble nutrients, especially sugars and amino acids, leaching from the roots (Bolton *et al*., 1993), but also from the mucilage produced by the root cap and from sloughed maize (Campbell and Greaves, 1990). Johansen and Binnerup (2002) found that CFB (*Cytophaga-Flavobacterium-Bacteroides*) and fluorescent *Pseudomonas* were two taxonomic groups that occurred abundantly in the rhizosphere of barley plants and were important contributors to the recycling of organic matter in the soil.

Other flavobacteria found in soil that could possibly come into contact with vegetable foodstuffs, include *F. cucumis, F. terrae,* (Weon *et al.,* 2007) and *F. daejeonense* and *F. suncheonense* (Kim *et al*., 2006) from greenhouse soils.

2.5.7 Drinking water

Water is a potential source of microbial contamination of food since it comes into contact with foodstuffs during the production, harvesting and processing of food raw materials (Leclerc and Moreau, 2002). Environmental flavobacterial strains have been isolated from a variety of water and marine environments (Floodgate and Hayes, 1963; Mudarris and Austin, 1989). Flavobacteria have also been encountered in various water purification systems, as well as in activated sludge plants where they represented up to 60% of the bacterial population in these environments. Members of the CDC Group IIb, now described as *Chryseobacterium* species, were reportedly

isolated from meat; but it was speculated that the actual source of these organisms was river water (Hayes, 1977).

Several *Chryseobacterium* strains were isolated from the groundwater of a municipal water supply in Germany (Ultee *et al*., 2004). According to Pavlov *et al*. (2004), *Chryseobacterium* species were also among the most common bacteria isolated from samples of treated and untreated drinking water in South Africa. A strain of *C. hispanicum* was isolated from the drinking water distribution system in Seville, Spain (Gallego *et al*., 2006). Two strains of *C. aquaticum* were isolated from a water reservoir in Korea (Kim *et al.*, 2008).

2.7 Conclusions

The taxonomy of the family *Flavobacteriaceae* has evolved rapidly since its inception in 1985. It is especially in the past 10 to 15 years that the numbers of genera in this family have increased from about 10 to 76 at the date of writing. Only 10 of these genera are, however, associated with food. This study gives a brief overview of the present state of the taxonomy of the *Flavobacteriaceae* family and also describes the variety of habitats in which the members of this family may occur. An overview of research on the food flavobacteria performed at the University of the Free State was presented since it gives a background as to why this study was undertaken. Flavobacteria are found in a variety of food sources which has been referred to in the latter section of the literature review. The spoilage characteristics of the flavobacteria were also discussed in this section. It became clear that the flavobacteria are in many cases food spoilage organisms since they not only have the ability to grow at low temperatures, but can also produce spoilage enzymes which have an effect on the texture, colour and odour of the food product.

CHAPTER 3

MATERIALS AND METHODS

In this study, flavobacterial isolates from different food sources were evaluated for their occurrence and spoilage characteristics. The first group of organisms was isolated from vegetables and/or soil surrounding the specific vegetable. Although a few members of the *Flavobacteriaceae* have been isolated from vegetables and the soil environment in studies globally (see Chapter 2), no study in South Africa has yet examined this ecological source for the presence of these bacteria.

The second group of bacteria consisted of dairy isolates already present in the University of the Free State Bacterial Culture Collection (UFSBC). These isolates were identified by phenotypic tests, using the BIOLOG system and by genetic means, using 16S rRNA sequencing. The BIOLOG system was also employed to determine the food spoilage potential of these isolates.

3.1 Isolation of *Flavobacteriaceae* isolates from vegetables and soil

3.1.1 *Sample collection*

Vegetables (e.g. beetroot, cabbage, carrots, lettuce and more) and/or soil surrounding the vegetables were collected from different locations in the Free State, South Africa. These locations and vegetables are stipulated in Table 3.1. Subsequent to sample collection, the samples in sterile stomacher bags were put in a cooler box with ice blocks and transported to the laboratory where they were kept at 4 ^{o}C until further analysis within 48 h.

Table 3.1. Sources of isolation of yellow pigmented Gram-negative isolates from vegetables and soil

Area	Commodity
Theunissen	Cabbage and surrounding soil
	Spinach and surrounding soil
	Sweet potato and surrounding soil
	Carrot and surrounding soil
Bloemfontein (Fruit & Vegetable city)	Beetroot
	Sweet potato
	Mushroom
Virginia	Spinach and surrounding soil
	Lettuce and surrounding soil

3.1.2 *Isolation and maintenance of yellow-pigmented colonies*

From each sample, 10 g of each of the vegetables and soil were weighed into separate sterile stomacher bags (Whirl-Pak™) and homogenised for 1-2 minutes in 90 ml 1 N phosphate buffer using a Stomacher Lab Blender 400 (ART Medical Equipment). Serial dilutions were aseptically prepared and plated onto nutrient agar (NA; Oxoid CM003) and the plates were then incubated at 25 ˚C for 48 h. Single yellow-pigmented colonies on the plates were picked and streaked onto brilliant green agar (BGA; Oxoid CM0329) to eliminate any Gram-positive yellow-pigmented organisms. Colonies from the BGA plates were re-streaked onto nutrient agar until purified. Gram staining using the Lillie's modification (Cowan, 1974), was then performed to confirm the purity of the cultures.

Short-term maintenance of the purified isolates was on nutrient agar slants which were kept at 4 ºC and which were re-streaked every 4-6 weeks. Long-term preservation of the isolates was done by freeze-drying and preservation at -20 ºC.

3.1.3 *Phenotypic characterization of the yellow-pigmented isolates*

A range of phenotypic tests, which also confirmed that the isolates belonged to the *Flavobacteriaceae* family, were performed on 601, 24 h old, yellow-pigmented, Gram-negative cultures from the above mentioned sources: these tests were performed according to the methods of MacFaddin (1980) and incubated at 25 °C, unless otherwise stipulated and included i) the production of catalase, phosphatase and oxidase enzymes; ii) the production of flexirubin-pigments by flooding a small mass of bacterial cells on a glass slide on a white background with 20% (w/v) potassium hydroxide. When the colour changed from yellow to red, purple or brown, it was regarded as flexirubin positive (Reichenbach, 1989); iii) Motility by examining culture suspensions by using phase-contrast microscopy. These suspensions were 24 h cultures in nutrient broth (Oxoid CM67); iv) hydrolysis of esculin incubated for 5 days; DNase production incubated for 2 days and v) gelatine hydrolysis for 36 h. For the determination of the latter hydrolytic activities, multi-inoculation of the media using standardized cell suspensions was performed with a multi-inoculation device (Jooste, 1985). The standardization of the suspension entailed suspending growth from a 24 h nutrient agar slant culture in 5 ml of sterile phosphate buffer (1 N) until a density comparable to a McFarland no. 2 standard (Difco 0691326) had been attained.

3.1.4 *Utilisation of carbon sources and identification of isolates*

The isolates were streaked onto nutrient agar plates and incubated at 25 °C for 24 h. Once again Gram stain, oxidase and catalase tests were performed to verify that the isolates were Gram-negative, oxidase and catalase positive. The isolates were then inoculated onto Triple Sugar Iron (TSI) agar slants as described by Fankhauser (2001). The isolates were subjected to testing on BIOLOG GN2 Microplates (Biolog, Inc., Hayward, California) according to the manufacturer’s protocol to identify the isolates and to determine the utilization of the carbon sources. The GN-NENT protocol was followed if the isolate was oxidase positive and had an

alkaline/alkaline (K/K) or alkaline/acid (K/A) reaction on a TSI slant. The isolates in this study fell in this group and were subsequently streaked out onto BIOLOG Universal Growth Agar (BUG) and incubated at 25 °C for 24 h. For identification, the GN2 Microplates were visually read (well by well) after 16 h and 24 h of incubation at 25 °C.

3.2 Re-examination and identification of dairy isolates from the UFSBC

3.2.1 *Revival of freeze-dried dairy isolates*

The 57 isolates for this part of the study were isolated from raw milk and butter samples from diverse regions of South Africa in a previous study (Jooste, 1985) and are shown in Table 3.2 (a to d). The isolates were maintained in a freeze-dried state and kept at -20 °C. These freeze-dried cultures were revived by inoculation into Nutrient Broth (NB; Oxoid CM67) and incubated for 48 h at 25 °C. This was followed by streaking onto Nutrient Agar (Oxoid CM003) and incubation for 48 h at 25 °C. For verification of pure cultures, Gram staining with the Lillie's modification (Cowan, 1974) was performed on all the cultures before further analysis.

3.2.2 *Phenotypic characterization of the dairy isolates*

The 57 dairy isolates were subjected to the following phenotypic tests. Motility was determined by preparing wet mounts of the isolates from 24 h old nutrient broth cultures grown at 25 °C and were examined by phase-contrast microscopy. A range of biochemical tests according to MacFaddin (1980), Gerhardt *et al*. (1981) and Barrow and Feltham (1993) and as suggested by Bernardet *et al.* (2002) were determined on 24 h standardised suspensions. The suspensions were standardised against MacFarland no. 2 standards as described in 3.1.3. These tests included: growth in 0% to 5% (w/v) sodium chloride; growth at 5 °C, 25 °C, 37 °C and 42 °C; growth on β-hydroxybutyrate agar and MacConkey agar (Oxoid CM115); production of catalase, oxidase, phosphatase, DNase, urease, indole (Kovacs reagent), β-galactosidase (ONPG) and nitrate reduction;

Table 3.2(a) *Chryseobacterium balustinum*-**like dairy isolates from the study of Jooste (1985) used in this study. (*, isolates used for 16S rRNA sequencing).**

Isolate No. (This study)	Isolate code (Jooste, 1985)	Source and region of isolation
1	Bf4a	Raw milk, Balfour
2	CT6a	Raw milk, Cape Peninsula
3	Eh2a	Raw milk, Eikenhof
4	Hb1a	Raw milk, Heidelberg
5	Hb2b	Raw milk, Heidelberg
6	Hb1b	Raw milk, Heidelberg
7*	Hb8b	Raw milk, Heidelberg
8	Hbg9a	Raw milk, Heidelberg
9	K7	Raw milk, Koppies
10	K10-1	Raw milk, Koppies
11	K17-1	Raw milk, Koppies
12*	MK4	Raw milk, Middelburg-Kriel
13*	MK5	Raw milk, Middelburg-Kriel
14*	MK2b	Raw milk, Middelburg-Kriel
15	NG7a	Raw milk, Nigel
16	SS16	Raw milk, Steenkoolspruit
17*	PE17	Raw milk, Port Elizabeth
18	Vv4a	Raw milk, Verkeerdevlei
19	76-2	Raw milk, Bloemfontein

Table 3.2(b) *Empedobacter*-**like dairy isolates from the study of Jooste (1985) used in this study. (*, isolates used for 16S rRNA sequencing).**

Isolate No. (This study)	Isolate code (Jooste, 1985)	Source and region of isolation
20	CT13c	Raw milk, Cape Peninsula
21	CT25d	Raw milk, Cape Peninsula
22*	Hbg2b	Raw milk, Heidelberg route
23	Hb5a	Raw milk, Heidelberg
24	Sp-6b	Raw milk, Sasol-Parys
25*	SS10a	Raw milk, Steenkoolspruit
26	78.3	Raw milk, Bloemfontein
27*	83.1	Raw milk, Bloemfontein

Table 3.2(c) *Weeksella*-**like dairy isolates from the study of Jooste (1985) used in this study. (*, isolates used for 16S rRNA sequencing).**

Isolate No. (This study)	Isolate code (Jooste, 1985)	Source and region of isolation
28	D215	Butter, Winburg
29*	D413	Butter, Bloemfontein
30*	Dn13a	Raw milk, Balfour (Wits)
31	Dn13c	Raw milk, Devon
32	E212	Butter, Winburg
33	Eh1a	Raw milk, Eikenhof
34	Eh2b	Raw milk, Eikenhof
35*	G222	Butter, Winburg
36	H31	Butter, Kimberley
37	H310	Butter, Kimberley
38	H329	Butter, Kimberley
39	H611	Butter, Ladysmith
40*	Hbg11a	Raw milk, Heidelberg route
41	J33	Butter, Kimberley
42	K323	Butter, Kimberley
43*	NG7b	Raw milk, Nigel (Wits)
44*	NG8a	Raw milk, Nigel (Wits)
45	NG12a	Raw milk, Nigel (Wits)
46	NG6	Raw milk, Nigel (Wits)
47*	74.1	Raw milk, Bloemfontein

Table 3.2(d) Unidentified dairy isolates from the study of Jooste (1985) and Hugo (1997) used in this study. (*, isolates used for 16S rRNA sequencing).

Isolate No. (This study)	Isolate code (Jooste, 1985)	Source and region of isolation
48	CT1a	Raw milk, Cape Peninsula
49	CT20a	Raw milk, Cape Peninsula
50*	Dn2b	Raw milk, Devon
51	Dn5a	Raw milk, Devon
52	Hbg13d	Raw milk, Heidelberg route (Wits)
53*	MK8a	Raw milk, Middelburg-Kriel
54	PE3c	Raw milk, Port Elizabeth
55	PE18a	Raw milk, Port Elizabeth
56	Vv2b	Raw milk, Verkeerdevlei
57*	Vv5a	Raw milk, Verkeerdevlei

hydrolysis of esculin, gelatine (tube method), and starch; and acid production from carbohydrates in ammonium salts medium containing the following sugars: arabinose, cellobiose, D-fructose, D-glucose, lactose, maltose, mannitol, rhamnose, sorbitol, sucrose, trehalose, and D-xylose (Hugo *et al*., 2003).

The isolates were also tested for their susceptibility to penicillin G (Pen G) using the Kirby-Bauer Disk Diffusion Method. Standardized (MacFarland no. 2) bacterial suspensions of the isolates were prepared. Using a sterile cotton swab, the above suspensions were then streaked out over an entire plate of Mueller-Hinton Agar (Oxoid CM337) in three different directions. Using sterile forceps, Pen G antimicrobial disks were placed onto the inoculated plates. The plates were then incubated for 48 h at 25 ^{o}C. After incubation the diameters of the zones of clearance around each disc were measured.

3.2.3 Utilisation of carbon sources and identification of isolates

The same procedure was followed with the 57 dairy isolates as with the vegetable and soil isolates (see 3.1.4).

3.3 16S rRNA sequencing

Polymerase Chain Reaction (PCR) amplification of the 16S rDNA region was performed on extracted DNA from 18 dairy isolates in this study (Table 3.2 (a to d). The 18 isolates were representative and randomly selected from different groups and sources. The method by Labuschagne and Albertyn (2007) was used to extract the DNA.

The reaction mixture contained PCR buffer (15 mM $MgCl_2$,) the forward (27F) and reverse primers (1492R), deoxyribonucleotide triphosphate (dNTP's), the cell suspension and double distilled water. Thermal cycling was performed using an Eppendorf Mastercycler Temperature Gradient Personal thermal cycler with the following cycling program steps: initial denaturing at 95 ˚C for 5 min, 35 cycles of denaturing at 94 ˚C for 15 sec, annealing at 50 ˚C for 30 sec and elongation at 72 ˚C for 90 sec. After 35 cycles, a final elongation step of 20 min at 72 ˚C was added to ensure complete elongation of the amplified product.

The PCR product was visualized on a 1% (w/v) agarose gel containing 0.5 µg/ml (final concentration) ethidium bromide. The agarose gels were prepared in TAE buffer containing 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. The PCR product was purified using the Zymo Research DNA clean and concentrator kit according to the manufactures' protocol. Sequenced using both the forward (27F) and reverse primers (1492R) with a BigDye® Terminator v1.1 sequence cycler according to the manufacturer's instructions. A phylogenetic tree was constructed using the neighbour-joining method in

the MEGA 4 software (Kumar *et al*., 2008), to determine the relationship of isolates under study and those on the database.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Taxonomic data

4.1.1 Vegetable and soil isolates

4.1.1.1 General phenotypic classification

A total of 601 Gram-negative yellow pigmented rods were isolated from the seven different vegetables and surrounding soil referred to in Table 3.1. When further phenotypic tests used to identify isolates belonging to the *Flavobacteriaceae* family were applied, only ten isolates were found to belong in this family (Table 4.1). These 10 isolates were recovered from the mushrooms and carrots as well as from the soil surrounding the lettuce plant (Table 4.2).

Table 4.1 Differential phenotypic characteristics of the 10 vegetable and soil isolates from the Free State region. (+, positive reaction; -, negative reaction; V, vegetable; S, soil).

Characteristic	V1	V2	V3	V4	V5	V6	V7	V8	S9	S10
Flexirubin pigment	-	-	-	-	-	-	-	-	+	+
Motility	-	-	-	-	-	-	-	-	-	-
Production of:										
DNase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Phosphatase	+	+	+	+	+	+	+	+	+	+
Degradation of:										
Esculin	+	+	+	+	+	+	+	+	+	+
Gelatine	+	-	+	-	+	+	+	+	+	+

All the isolates were non-motile and were able to produce DNase, catalase, oxidase, phosphatase and degrade esculin. Isolates S9 and S10 were the only isolates that produced flexirubin pigments. Isolates V2 and V4 were not able to degrade gelatine while all the other isolates exhibited this characteristic.

After the phenotypic confirmation that the isolates belong in the *Flavobacteriaceae* family, the isolates were subjected to BIOLOG characterisation. The results obtained after 16 h of incubation can be seen in Table 4.2a and those obtained after 24 h incubation in Table 4.2b.

4.1.1.2 Phenotypic identification using the BIOLOG system

According to Biolog Inc., the similarity index (SIM) at 16 h to 24 h of incubation must be at least 0.50 to yield an acceptable identification profile. The SIM is determined by Biolog's MicroLog computer software. It was clear from the results in Table 4.2a that isolate numbers V1, V5, V6 and V8 could not yet be identified by the BIOLOG system at 16 h of incubation, having SIM values of less than 0.50. Adequate and stable identification patterns on the BIOLOG MicroPlates were, however, formed after 24 h of incubation resulting in the SIM values exceeding 0.50. The reading of BIOLOG MicroPlates in this study was, therefore, regarded as more accurate at 24 h of incubation. This is also confirmed by the higher percentage of probability values (between 90 and 100%) for all 10 isolates.

Table 4.2a Identification of the 10 vegetable and soil isolates with the BIOLOG system after 16 h of incubation. (-, no results; *C.*, *Chryseobacterium*; *S.*, *Sphingobacterium*; SIM, similarity index; * isolates used for 16S rRNA sequencing).

Isolate number	Source	Probability (%)	SIM index	Genus/Species identification
V1	Mushrooms	-	0.23	-
V2	Mushrooms	76	0.59	*S. mizutaii*
V3	Mushrooms	89	0.51	*S. mizutaii*
V4	Mushrooms	84	0.67	*S. mizutaii*
V5	Mushrooms	-	0.35	*Flavobacterium*
V6	Mushrooms	-	0.44	*Flavobacterium*
V7	Carrots	94	0.66	*S. mizutaii*
V8	Carrots	-	0.43	*Sphingomonas*
S9*	Lettuce/soil	95	0.50	*C. gleum/indologenes*
S10*	Lettuce/soil	100	0.68	*C. gleum/indologenes*

Table 4.2b Identification of the 10 vegetable and soil isolates with the BIOLOG system after 24 h of incubation. (*C.*, *Chryseobacterium*; *S.*, *Sphingobacterium*; SIM, similarity index; * isolates used for 16S rRNA sequencing).

Isolate number	Source	Probability (%)	SIM index	Genus/Species identification
V1	Mushrooms	95	0.63	*S. thalpophilum*
V2	Mushrooms	93	0.61	*S. mizutaii*
V3	Mushrooms	99	0.71	*S. mizutaii*
V4	Mushrooms	90	0.56	*S. mizutaii*
V5	Mushrooms	94	0.73	*S. mizutaii*
V6	Mushrooms	97	0.69	*S. mizutaii*
V7	Carrots	94	0.66	*S. mizutaii*
V8	Carrots	93	0.61	*S. multivorum*
S9*	Lettuce/soil	97	0.56	*C. gleum/indologenes*
S10*	Lettuce/soil	100	0.68	*C. gleum/indologenes*

From the BIOLOG results, isolate numbers V1 to V8 were identified as *Sphingobacterium* species, with isolate V1 as *S. thalpophilum*, isolate V2 to V7 as *S. mizutaii* and isolate V8 as *S. multivorum*. The genus *Sphingobacterium* was first proposed by Yabuuchi *et al*. (1983) and formed part of the family *Flavobacteriaceae*. In 1998, however, the *Sphingobacteriaceae* family was established (Steyn *et al*., 1998). Although the sphingobacteria are also Gram-negative rods which produce yellow-pigmented colonies, they can be differentiated from the flavobacteria in that they possess sphingolipids, typically have a MK-7 quinone respiratory system, higher mean G + C contents in the DNA and a unique cellular fatty acid composition (Steyn *et al.*, 1998).

Sphingobacterium multivorum, S. mizutaii and *S. thalpophilum* have been isolated from clinical sources (Holmes *et al*., 1983; Yabuuchi *et al.*, 1983). *Sphingobacterium multivorum* has also been associated with causing cystic fibrosis (Reina *et al.*, 1992) and septicaemia (Freney *et al.,* 1987). Currently, the *Sphingobacterium* genus consists of 12 species (Euzéby, 2008). Most of these species are frequently isolated from soil, e.g. *Sphingobacterium anhuiense* from forest soil (Wei *et al*., 2008), *S. canadense* from corn roots (Samina *et al*., 2007), *S. daejeonse* from compost (Kim *et al*., 2006), and *S. composti* from cotton-waste compost which was used as medium for the cultivation of oyster mushrooms (Yoon *et al*., 2007), *Sphingobacterium kitahiroshimense* was isolated from soil (Matsuyama *et al*., 2008), and *Sphingobacterium siyangense* from farm soil (Liu *et al*., 2008).

In this study, eight *Sphingobacterium* strains were isolated mainly from mushrooms, but also from carrots. It is speculated that the source of these bacteria is the soil surrounding the mushrooms and carrots. Although the 8 strains were identified as belonging to *S. multivorum, S. thalpophilum* and *S. mizutaii*, the percentage probability of identification values were not 100% which is an indication that these strains could belong to other more recently described *Sphingobacterium* species. The BIOLOG database used in this study was developed in 2001 and could only identify *S. mizutaii, S.*

multivorum, S. spiritivorum and *S. thalpophilum*. The information on the newer species of *Sphingobacterium* described in the previous paragraph are yet not included in the BIOLOG database.

Isolate numbers S9 and S10 were both identified as *Chryseobacterium gleum/indologenes* respectively. The phenotypic characteristics of these two isolates conformed to the genus *Chryseobacterium*. According to Bernardet *et al*. (2006), the absence of gliding motility and the presence of flexirubin type pigments in members of the genus *Chryseobacterium* are two of the key characteristics which differentiate them from other members of the family *Flavobacteriaceae*. The BIOLOG database (2001) used in this study was, however, not capable of differentiating between these two closely related *Chryseobacterium* species. Isolate S10 could with certainty (100% probability) be identified as belonging to either *C. gleum* or *C. indologenes*. Isolate S9, however, had a probability value of 97%. This is an indication that this isolate may belong to another more newly described *Chryseobacterium* species currently not in the BIOLOG database that was used in this study. The database only contained five *Chryseobacterium* species, namely *C. balustinum, C. gleum/indologenes, C. indoltheticum* and *C. scophthalmum*, while this genus currently consists of 35 species (Euzéby, 2008).

Isolates S9 and S10 were both isolated from the rhizosphere of the lettuce. This is not the first time that the *Chryseobacterium* genus has been isolated from this source. A strain of *Chryseobacterium formosense* was isolated from the rhizosphere of garden lettuce (*Lactuca sativa*) in Taiwan (Young *et al*., 2005) while *C. wanjuense* was also isolated from greenhouse soil in which lettuce (*Lactuca sativa*) was cultivated (Weon *et al*., 2006).

In general, it is not surprising to find bacteria on vegetables and in soil, because soil is the natural habitat of many microorganisms (Banwart, 1989). Some microorganisms do not attach to plants and are not capable of proliferating on vegetables (Tournas, 2005). They may however find some ecological advantage in the rhizosphere of the specific plant. This could be

the reason for the affinity of the *Chryseobacterium* isolates for the environment of the lettuce root system.

4.1.1.3 Genetic identification using 16S rRNA sequencing

The 16S rRNA sequences of the 10 vegetable and soil isolates unfortunately were of a poor quantity and no results could, therefore, be obtained. The DNA concentration for the vegetable and soil samples was in the range between 8-10 ng/µl when the amount of DNA was checked for all the samples on the Nanodrop Spectrophotometer before sequencing. Again, 2 µl of the genomic DNA after cleaning the PCR product, was run on a gel to check the density of the DNA, the DNA bands for the vegetable and soil samples were faint. Presumably some DNA was lost after cleaning the PCR product using the Zymo Research DNA Clean and Concentrator for these samples. This was a learning process, because ultimately, the results for the dairy samples improved, but unfortunately the vegetable and soil samples results failed after three attempts. The quantity of the DNA template for sequencing is crucial and is a common problem when sequencing the PCR product. Too little DNA template reduces the signal length and peak height, and this was detected with these samples (Clarridge, 2004).

4.1.2 Dairy isolates

4.1.2.1 General phenotypic classification and identification

Fifty seven dairy isolates from the study of Jooste (1985) were used for this part of the study. These isolates could not be identified with certainty in the study of Jooste which employed numerical phenotypic analysis and chemotaxonomic techniques. As a result the isolates were assigned to groups that resembled the *Chryseobacterium, Weeksella* and *Empedobacter* genera. There was also an unidentified group of isolates that could not be placed in any of the known *Flavobacteriaceae* genera at that time (1985). In a later study (Hugo, 1997), this last group formed a cluster during SDS-PAGE

analysis, but still could not be assigned to specific *Flavobacteriaceae* genera. In this study, these four groups were evaluated for newer phenotypic characteristics as recommended by the minimal standards for *Flavobacteriaceae* members (Bernardet *et al*., 2002). They were also subjected to BIOLOG analysis and 16S rRNA sequencing for correct identification. After identification, the potential spoilage characteristics of the identified different genera and species were deduced using the BIOLOG system.

Chryseobacterium balustinum-like isolates

The phenotypic characteristics of the 19 isolates in this group are shown in Table 4.3. All 19 isolates were strictly aerobic, Gram-negative rods. After 24 h growth on Nutrient Agar, all the colonies, except one isolate, were yellow pigmented producing a flexirubin pigment (Table 4.3). All isolates were positive for the production of catalase, oxidase and DNase; hydrolysis of gelatine; growth at 25 ˚C; and growth on β-hydroxybutyrate agar. The growth on MacConkey Agar varied amongst isolates which is in accordance with Bernardet *et al*. (2006) who found that the ability to grow on MacConkey agar varies among *Chryseobacterium* species. All isolates were negative for motility; growth at 42 ˚C; growth in 4% and 5% NaCl (w/v); acid production from arabinose, mannitol, sorbitol and xylose. While *Chryseobacterium* species are able to oxidize some carbohydrates, several of them are unable to produce acid from carbohydrates (Bernardet *et al*., 2006). This feature is considered a differentiating characteristic between *Chryseobacterium* species.

Weeksella-like isolates

The phenotypic characteristics of this group are presented in Table 4.4. All 20 isolates in this group were strictly aerobic Gram-negative rods. All isolates were positive for the production of catalase; growth at 25 ˚C; and

Table 4.3 Phenotypic characteristics of the *Chryseobacterium balustinum***-like dairy isolates from the study of Jooste (1985).**

(F, flexirubin; +, positive reaction; -, negative reaction; +/-, weakly positive**)**.

	Isolate numbers																		
Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Pigment	F	F	F	F	F	F	F	F	F	F	F	F	F	F	-	F	F	F	F
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of:																			
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Indole	+	-	-	+	-	-	+	+	+	+	+	+	+	-	+	+	-	+	-
Phosphatase	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
DNase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
β-galactosidase (ONGP)	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Susceptibility to Pen G	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Degradation of:																			
Starch	+	+	+	+	-	+	+	+	-	+	-	+	+	-	-	+	+	+	-
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 4.3 continued.

	Isolate numbers																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Growth at:																			
25 ˚C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 ˚C	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-
42 ˚C	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Growth in NaCI (w/v):																			
1 %	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
2 %	+	-	-	+	+	+	+/-	+	-	-	-	+	+	+	-	-	+	+	+/-
3 %	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
4 %	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
5 %	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Growth on:																			
MacConkey Agar	-	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
β- Hydroxybutyrate Agar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from sugars:																			
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Fructose		-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
Glucose	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-	-
Maltose	+/-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	+/-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	+/-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+/-	+/-	+/-	-	-	-	+/-	-	-	+/-	+/-	-	+/-	+/-	-	-	-	-	-
Trehalose	-	+/-	+/-	-	-	-	-	+/-	-	-	-	-	+/-	-	-	-	-	+/-	-

Table 4.4 Phenotypic characteristics of the *Weeksella*-like dairy isolates from the study of Jooste (1985). (F, flexirubin; +, positive reaction; -, negative reaction; +/-, weakly positive**)**.

	Isolate numbers																			
Characteristic	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**
Pigment	-	-	-	-	-	F	F	-	-	-	-	-	-	-	-	-	-	F	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of:																				
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Indole	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	+	-	-	-	-
Phosphatase	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	-	+
DNase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Nitrate reduction	+	+	-	-	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-
β-galactosidase (ONGP)	-	+	-	-	-	-		+	-	-	+	-	-	-	-	-	-	-	-	-
Susceptibility to Pen G	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+
Degradation of:																				
Starch	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-
Esculin	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+
Gelatin	-	+	-	+	-	+	-	-	-	-	-	-	+	+	-	+	+	+	+	+
Growth at:																				
25 ˚C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 ˚C	-	-	+	+	-	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-
42 ˚C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Isolate numbers																			
Characteristic	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**
Growth on NaCl (w/v):																				
1 %	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2 %	-	+	+	-	+	-	+	+	-	+	+/-	+	-	-	-	-	-	+	+	+/-
3 %	-	+	-	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	+	-	-
4 %	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 %	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on:																				
MacConkey Agar	+/-	+	-	+	+	-	-	-	-	+/-	+/-	+/-	-	+	+/-	-	-	+	+	+
β-Hydroxybutyrate Agar	+	+	+	+/-	-	+	+/-	-	+/-	-	-	-	-	-	-	+	+	+	+	-
Acid from sugars:																				
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	+/-	+/-	-	+/-	-
Glucose	-	+/-	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-	-	-	+/-	+/-	+/-	-	+/-
Lactose	+/-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	+/-	+/-	-	+/-	+/-
Mannitol	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-
Trehalose	+/-	+/-	+/-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	+/-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

growth in 1% NaCI (w/v). All the isolates were negative for motility; acid production from arabinose, rhamnose, sorbitol and xylose. All isolates reported as *Weeksella*-like were yellow pigmented when grown on Nutrient Agar at 25 ˚C for 24 h. However, according to Tatum *et al*. (1974) and von Graevenitz (1995) *Weeksella* colonies are tan to brown in colour. The majority of these isolates were unable to produce the flexirubin type of pigment, except three isolates (Table 4.4). According to Hugo *et al*. (2006), the genus *Weeksella* does not produce DNase, urease and β-galactosidase, and does not reduce nitrate. They are non-saccharolytic and do not degrade starch, esculin or agar. On the contrary, some *Weeksella*-like isolates in this study produced DNase, urease and β-galactosidase. A few isolates reduced nitrate, and weakly produced acid from some of the carbohydrates tested.

Empedobacter-like isolates

The phenotypic characteristics of the isolates in this group are given in Table 4.5. All eight isolates were strictly aerobic, Gram-negative rods, and yellow pigmented when grown on nutrient agar for 24 h. However, not all isolates were able to produce a flexirubin type pigment (Table 4.5). Bernardet *et al*. (2002) found that the yellow pigment produced by the members of the family *Flavobacteriaceae* may belong to the carotenoid or to the flexirubin type depending on the genus. Therefore we presume that all the *Empedobacter*-like isolates which did not produce a flexirubin pigment, could have produced a carotenoid type of pigment. All isolates were positive for the production of catalase, oxidase, phosphatase and DNase; growth in 1% and 2% NaCI (w/v); growth at 25 ˚C and 37 ˚C and degradation of gelatine. All the isolates were negative for motility; growth at 4% and 5% NaCl (w/v); hydrolysis of starch and acid production from arabinose, cellobiose, lactose, mannitol, rhamnose and sorbitol. Although Hugo *et al*. (2006) stated that the genus *Empedobacter* is negative for the production of β-galactosidase, nitrate reduction and hydrolysis of esculin, some *Empedobacter*-like isolates in this study were

Table 4.5 Phenotypic characteristics of the *Empedobacter*-like dairy isolates from the study of Jooste (1985). (F, flexirubin; +, positive reaction; -, negative reaction; +/-, weakly positive**)**.

	Isolate numbers							
	40	**41**	**42**	**43**	**44**	**45**	**46**	**47**
Pigment	F	F	F	F	-	F	-	F
Motility	-	-	-	-	-	-	-	-
Production of:								
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
Urease	-	-	-	+	+	-	-	-
Indole	+	-	-	-	-	-	-	-
Phosphatase	+	+	+	+	+	+	+	+
DNase	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	-	-	-	+	-	-
β-galactosidase (ONGP)	-	-	-	-	-	+	-	-
Susceptibility to pen G	-	-	-	-	-	+	-	-
Degradation of:								
Starch	-	-	-	-	-	-	-	-
Esculin	+	+	+	-	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+
Growth at:								
25 ˚C	+	+	+	+	+	+	+	+
37 ˚C	+	+	+	+	+	+	+	+
42 ˚C	-	-	-	+	+	-	-	-
Growth in NaCl (w/v):								
1 %	+	+	+	+	+	+	+	+
2 %	+	+	+	+	+	+	+	+
3 %	+	+	+	-	-	-	+	+
4 %	-	-	-	-	-	-	-	-
5 %	-	-	-	-	-	-	-	-
Growth on:								
MacConkey Agar	+	+	-	+	+	+/-	+	+
β-Hydroxybutyrate Agar	+	+	+	+	+	+	+/-	-
Acid from sugars:								
Arabinose	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-
Fructose	-	-	+/-	-	-	-	-	-
Glucose	+/-	+/-	+/-	+/-	+/-	+/-	-	-
Lactose	-	-	-	-	-	-	-	-
Maltose	-	-	+/-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
Trehalose	-	-	-	+/-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-

positive for the above mentioned characteristics. The tests in which the isolates gave variable results are illustrated in Table 4.5.

Unidentified SDS-PAGE isolates from the study of Jooste (1985) and Hugo (1997)

The results of the phenotypic characteristics of this unidentified group are presented in Table 4.6. All 10 isolates were strictly aerobic, Gram-negative rods. All the isolates were positive for the production of flexirubin type of pigment; catalase, oxidase and DNase; growth at 25 ˚C and 37 ˚C; growth in 1% and 2% NaCI and hydrolysis of gelatine and esculin. All the isolates were negative for motility, β-galactosidase, susceptibility to Pen G; growth in 5% NaCI; acid production from cellobiose, fructose, lactose, maltose, rhamnose, sorbitol and xylose.

Phenotypic variability among strains belonging to the same species sometimes results in bacterial isolates presenting characteristics that are atypical for isolate identification. Drancourt *et al*. (2000) found that in such cases it is essential that 16S rRNA based identification be applied, because it offers a useful alternative when phenotypic characterisation methods fail.

Table 4.6 Phenotypic characteristics of the unidentified SDS-PAGE group of the study of Hugo (1997). (F, flexirubin; +, positive reaction; -, negative reaction; +/-, weakly positive**)**.

	Isolate numbers									
Characteristic	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**
Pigment	F	F	F	F	F	F	F	F	F	F
Motility	-	-	-	-	-	-	-	-	-	-
Production of:										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Urease	+	-	+	+	+	+	+	+	+	+
Indole	+	+	-	+	-	-	-	-	-	-
Phosphatase	-	+	+	+	+	+	+	-	+	+
DNase	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	-	-	-	-	+	-	-	-
β-galactosidase (ONGP)	-	-	-	-	-	-	-	-	-	-
Susceptibility to Pen G	-	-	-	-	-	-	-	-	-	-
Degradation of:										
Starch	+	+	+	+	+	-	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+
Growth at:										
25 ˚C	+	+	+	+	+	+	+	+	+	+
37 ˚C	+	+	+	+	+	+	+	+	+	+
42 ˚C	-	-	-	-	-	-	-	-	+	-
Growth in NaCl (w/v):										
1 %	+	+	+	+	+	+	+	+	+	+
2 %	+	+	+	+	+	+	+	+	+	+
3 %	+	+	-	-	+	+	+	+	-	+
4 %	-	-	-	-	-	+	+	+	-	-
5 %	-	-	-	-	-	-	-	-	-	-

Table 4.6 continued

	Isolate numbers									
Characteristic	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**
Growth on:	+	+	+	+	+	+	+	+	+	+
MacConkey Agar	+	+	+	+	+	+	-	+	+	+
β-Hydroxybutyrate Agar	+/-	+/-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
Acid from sugars:										
Arabinose	-	-	-	-	-	-	-	+/-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	-	-	-	-	-
Glucose	+/-	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Lactose	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	+	-	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-
Sucrose	+/-	+/-	-	-	-	+/-	-	-	-	-
Trehalose	+/	+/-	-	+/	-	-	+/-	+/-	+/-	+/
Xylose	-	-	-	-	-	-	-	-	-	-

4.1.2.2 Phenotypic identification using the BIOLOG system

The identification of all dairy isolates in this section was based on results of the BIOLOG Microplates incubated for 24h, as mentioned previously (section 4.1.1.2). Results are more stable and accurate after this period of incubation.

Chryseobacterium balustinum-like isolates

Most of the isolates (13 from 19) in this group belonged to the genus *Chryseobacterium* (Table 4.7). Only isolate numbers 8 and 11 were identified as *C. balustinum*, which was in accordance with the study of Jooste (1985). *Chryseobacterium balustinum* was originally isolated from heart blood of freshwater fish (Holmes *et al*., 1984a).

Table 4.7 Identification of *Chryseobacterium balustinum***-like isolates with the BIOLOG system after 24h of incubation.** (PROB, probability; SIM, similarity; ID, identification; *C.*, *Chryseobacterium*; *, isolates used for 16S rRNA sequencing; -, no results).

Isolate number	Isolate code	PROB (%)	SIM	BIOLOG ID
1	Bf4a	-	-	-
2	CT6a	76	0.56	*Flavobacterium hydatis*
3	Eh2a	80	0.66	*C. gleum/indologenes*
4	Hb1a	93	0.64	*C. gleum/indologenes*
5	Hb2b	99	0.73	*C. meningosepticum*
6	Hb1b	96	0.77	*C. gleum/indologenes*
7*	Hb8b	100	0.81	*C. gleum/indologenes*
8	Hbg9a	99	0.91	*C. balustinum*
9	K7	-	-	-
10	K10-1	-	-	-
11	K17-1	92	0.62	*C. balustinum*
12*	MK4	83	0.57	*C. gleum/indologenes*
13*	MK5	91	0.67	*C. meningosepticum*
14*	MK2b	-	-	-
15	NG7a	-	-	-
16	SS16	99	0.74	*C. meningosepticum*
17*	PE17	86	0.72	*C. indoltheticum*
18	Vv4a	92	0.55	*C. meningosepticum*
19	76-2	89	0.57	*C. meningosepticum*

Isolate number 17 was identified as *C*. *indoltheticum*. This organism was first isolated from marine mud. It was discovered during a study aimed at retrieving chitin degrading bacteria from marine environments (Campbell and Williams, 1951).

Isolate numbers 3, 4, 6, 7 and 12 were identified as *Chryseobacterium gleum/indologenes*. These isolates may either belong to *C. gleum* or *C. indologenes* or even other species of *Chryseobacterium*. The 2001 version of the BIOLOG Microplate system was unable to differentiate between these *Chryseobacterium* species. *Chryseobacterium gleum* was first described by Holmes *et al*. (1984b) and was isolated from the hospital environment (Holmes *et al*., 1984b; Yabuuchi *et al*., 1990). This organism is, however, regularly isolated from the dairy environment (Jooste *et al*., 1985; Hugo and Jooste, 1997; Hugo *et al*., 1999). *Chryseobacterium indologenes* was first described in 1983 (Yabuuchi *et al*., 1983). This organism occurs in clinical and food environments (Yabuuchi *et al*., 1983; Jooste *et al*., 1985).

Isolate numbers 5, 13, 16, 18 and 19 belonged to *C. meningosepticum*. This species was recently transferred to a new genus namely *Elizabethkingia meningoseptica* (Kim *et al*., 2005).

Isolate numbers 1, 9, 10, 14 and 15 were not identified by this method. The possible reason for this was that the profiles did not match any known genera and species in the current database of the BIOLOG system. Isolate number 2 was identified as *Flavobacterium hydatis*. This organism was formerly known as *Cytophaga aquatilis* (Bernardet *et al*., 1996), and was isolated from the gills of a diseased salmon, but the pathogenicity thereof was not tested.

Empedobacter-like isolates

Seven of the eight isolates in this group were identified by the BIOLOG system as *Empedobacter brevis* (Table 4.8), which is in accordance with the study by Jooste (1985). Isolate number 25 was identified as a CDC group ll-E subgroup B strain which is currently unknown. The probability and similarity values were generally high for this group with isolate numbers 20 and 26 having a 100% probability. The genus *Empedobacter* consists of only one species, *Empedobacter brevis*. The strains were first isolated from clinical

sources, but were subsequently also found in the food environment (Engelbrecht *et al*., 1996; Hugo *et al*., 2006).

Table 4.8 Identification of *Empedobacter*-like isolates with the BIOLOG system after 24h of incubation. (PROB, probability; SIM, similarity; ID, identification; *E., Empedobacter;* *, isolates used for 16S rRNA sequencing; -, no results).

Isolate number	Isolate code	PROB (%)	SIM	BIOLOG ID
20	CT13c	100	0.86	*E*. *brevis*
21	CT25d	93	0.73	*E*. *brevis*
22*	Hbg2b	98	0.72	*E*. *brevis*
23	Hb5a	76	0.73	*E*. *brevis*
24	Sp-6b	96	0.90	*E*. *brevis*
25*	SS10a	99	0.77	CDC group ll-E subgroup B
26	78.3	100	0.94	*E*. *brevis*
27*	83.1	96	0.66	*E*. *brevis*

Weeksella-like isolates

Although the isolates in this group were identified as members of the *Flavobacteriaceae* family, not one of the isolates in this group was identified by the BIOLOG system as *Weeksella* isolates (Table 4.9). The reasons for this are discussed in section 4.8 in the comparison of phenotypic and genotypic results.

Isolate numbers 29, 41 and 43 were not identified by this identification method. Isolate numbers 28 and 44 were identified as *C. gleum/indologenes*. Ursing and Bruun (1991) proposed that the identification of *C. gleum* and *C. indologenes* should be based on genomic techniques to differentiate between the species, because they are so closely related.

Table 4.9 Identification of *Weeksella*-like isolates with the BIOLOG system after 24h of incubation. (PROB, probability; SIM, similarity; ID, identification; *C.*, *Chryseobacterium*; *E., Empedobacter*; *, isolates used for 16S rRNA sequencing; -, no results**)**.

Isolate number	Isolate code	PROB (%)	SIM	BIOLOG ID
28*	D215	88	0.51	*C. gleum/indologenes*
29	D413	-	-	-
30*	Dn13a	91	0.64	*C. scophthalmum*
31	Dn13c	97	0.83	*E. brevis*
32	E212	97	0.65	*C. indoltheticum*
33	Eh1a	65	0.53	*E. brevis*
34	Eh2b	92	0.51	CDC group II-E subgroup A
35*	G222	74	0.59	*E. brevis*
36	H31	74	0.53	*C. indoltheticum*
37	H310	94	0.72	*Riemerella anatipestifer*
38	H329	70	0.51	*C. indoltheticum*
39	H611	95	0.57	*C. indoltheticum*
40*	Hbg11a	84	0.65	CDC group II-E subgroup A
41	J33	-	-	-
42	K323	83	0.51	CDC group II-E subgroup A
43*	NG7b	-	-	-
44*	NG8a	91	0.59	*C. gleum/indologenes*
45	NG12a	87	0.77	*E. brevis*
46	NG6	94	0.91	*E. brevis*
47*	74.1	82	0.56	*C. indoltheticum*

Isolate numbers 32, 36, 38, 39 and 47 were identified as *C. indoltheticum* while isolate number 30 belonged to *C. scophthalmum*. This

species was originally isolated from the gills of diseased turbot (*Scophthalmus maximus*) (Mudarris *et al*., 1994).

Isolate numbers 34, 40 and 42 were identified as CDC group II-E subgroup A which is currently unknown. Isolate numbers 31, 33, 35, 45 and 46 were identified as *E. brevis*.

Isolate number 37 was identified as *Riemerella anatipestifer*. The primary source of this species is blood of a duck and was found to cause septicemic disease of domesticated ducklings (Segers *et al*., 1993a).

Unidentified SDS-PAGE isolates from the study of Jooste (1985) and Hugo (1997)

The majority of isolates (6/10) in this group belonged to the genus *Chryseobacterium* (Table 4.10). Isolate numbers 49 and 54 could not be identified by this method. Isolate numbers 48 and 50 were identified as *E. brevis.* Isolate numbers 51 and 56 were identified as *C. balustinum*, isolate number 52 belonged to *C. meningosepticum* and isolate numbers 53, 55 and 57 were identified as *C. gleum/indologenes*.

Table 4.10 Identification of unidentified SDS-PAGE isolates from the study of Jooste (1985) and Hugo (1997) with the BIOLOG system after 24h of incubation. (PROB, probability; SIM, similarity; ID, identification; *C.*, *Chryseobacterium*; *E., Empedobacter*; *, isolates used for 16S rRNA sequencing; -, no results**)**.

Isolate number	Isolate code	PROB (%)	SIM	BIOLOG ID
48	CT1a	85	0.78	*E. brevis*
49	CT20a	-	-	-
50*	Dn2b	85	0.78	*E. brevis*
51	Dn5a	95	0.82	*C. balustinum*
52	Hbg13d	84	0.53	*C. meningosepticum*
53*	MK8a	99	0.70	*C. gleum/indologenes*
54	PE3c	-	-	-
55	PE18a	100	0.81	*C. gleum/indologenes*
56	Vv2b	100	0.90	*C. balustinum*
57*	Vv5a	77	0.64	*C. gleum/indologenes*

4.1.2.3 Genetic identification of dairy isolates using 16S rRNA gene sequencing

For this part of the study, 18 of the dairy isolates were used from the four groups, *Chryseobacterium balustinum*-like, *Empedobacter*-like, and *Weeksella*-like and the unidentified SDS-PAGE isolates (Jooste, 1985; Hugo, 1997). The 16S rRNA gene sequencing method was applied because it is a powerful tool that has been used to trace phylogenetic relationships between bacteria and to identify bacteria from various sources, such as environmental or clinical species, (Mignard and Flandrois, 2006).

Tables 4.11 to 4.14 present the identification of dairy isolates using the 16S rRNA sequencing method sequencing the 16S rRNA gene with a size of 1,500bp, giving two possible nearest phylogenetic neighbours and percent similarities. The discussion of the results will only consider the phylogenetic neighbour with a species name.

The expected value (E-value) and percentage similarity (% similarity) are inseparable when identifying bacteria using the 16S rRNA sequencing technique. The % similarity is required to assign an isolate to a particular species or genus and the E-value is used as a convenient way to create a significance threshold when reporting % similarity results. The lower the E-value, or closer it is to zero the more 'significant' the match is. In Tables 4.11 to 4.14 the E-value for all the dairy isolates in this study was zero. The sequence size generated for all eighteen dairy isolates was in the range between (1422-1485 pb) with a query coverage of 98%. This percentage indicates that only 2% of the bases for all the samples were not sequenced. But 98% of the bases of the samples in this study were matching those obtained from the database.

The *Chryseobacterium balustinum*-like group consisted of 19 isolates, of which only five were subjected to 16S rRNA gene sequencing analysis. The nearly full-length 16S rRNA genes of isolate numbers 7, 12, 13, 14, 17 were compared to those submitted at the NCBI GenBank database (Table 4.11). Searches indicated that isolate numbers 12 and 14 showed 98% sequence similarities to *C. shigense* and *C. indoltheticum* respectively. Isolate number 13 was closely related to *C. bovis* with a sequence similarity of 94%, while isolate number 7 aligned most closely with *C. aquaticum* showing 96% sequence similarity. All these *C. balustinum*-like isolates were phylogenetically closely related to species of the genus *Chryseobacterium*. Isolate number 17, however, was closely related to *Empedobacter brevis* showing 97% sequence similarity.

Table 4.11 16S rRNA-based identification of *Chryseobacterium balustinum*-like isolates.

Isolate no	E-value	Accession no	Nearest phylogenetic neighbour	% similarity
7	0.0	AM748690	*Chryseobacterium aquaticum* strain 10-46^T partial 16S rRNA sequence	96%
		AM748691	*Chryseobacterium aquaticum* strain 10-106 partial 16S rRNA sequence	96%
12	0.0	AYA46846.1	*Chryseobacterium* sp. UOF CM1396 partial 16S rRNA sequence	98%
		AB193101.1	*Chryseobacterium shigense* partial 16S rRNA sequence	98%
13	0.0	EF204448.1	*Chryseobacterium bovis* strain H15 partial 16S rRNA sequence	94%
		EF204447.1	*Chryseobacterium bovis* strain H10 partial 16S rRNA sequence	94%
14	0.0	DQ30070.1	*Chryseobacterium* sp. CI03 partial 16S rRNA sequence	98%
		AY468448.1	*Chryseobacterium indoltheticum* strain LMG 4025 partial 16S rRNA sequence	98%
17	0.0	AM177497	*Empedobacter brevis* strain LMG 4011^T partial 16S rRNA sequence	97%
		AY582884	*Flavobacterium* sp. GXW15-4 partial 16S rRNA sequence	97%

The *Empedobacter*-like group consisted of eight isolates, but only three isolates were analysed by means of 16S rRNA gene sequencing (Table 4.12). Isolate numbers 22, 25 and 27 were all closely related to *E. brevis*, with sequence similarities of 98%, 97% and 96% respectively.

The *Weeksella*-like group consisted of 20 isolates from milk and butter. Only seven isolates were further investigated via 16S rRNA gene sequencing (Table 4.13). Again, no isolate could be identified as *Weeksella*, which is in conformance with the BIOLOG results and the general phenotypic results. All seven isolates were found to belong to the *Chryseobacterium* genus. Isolate numbers 35, 43, 28 and 30 were found to be closely related to *C. bovis* strains (H15 and H10) showing sequence similarities of 97%, 98% and 99%, respectively. Isolate number 40 was aligned most closely with *C. haifense* with a sequence similarity of 97% and isolate number 44 was related to *C. shigense* with sequence similarities of 99%.

Three of the 10 isolates in the unidentified SDS-PAGE group from the study of Jooste (1985) and Hugo (1997) were further investigated using the 16S rRNA gene sequencing method. Isolate numbers 50 and 53 indicated sequence similarities of 99% with *C. ureilyticum* and *C. indologenes,* respectively, and isolate number 57 was closely related to *C. ginsengisoli* with a low sequence similarity of 95% (Table 4.14).

The relationship of all the dairy isolates in this study and those obtained from the database is illustrated in Fig. 4.1. Isolate number 40 was phylogenetically closely related to *C. haifense* with a sequence similarity of 97%, showing a bootstrap value of 100%, while isolate number 7 formed a cluster with *C. aquaticum* with a sequence similarity of 96%. Respective sequence similarities of 97% and 98% between isolate numbers 28, 30, 35 and 43 and *C. bovis* (EF 204448) does not exclude a possible relationship at the species level with one of these taxa (Hantsis-Zacharov *et al*., 2008). However, isolate number 13 with a sequence similarity 94%, did not indicate relatedness at the species level to *C. bovis* (EF 204448).

Table 4.12 16S rRNA-based identification of *Empedobacter*-like isolates.

Isolate no	E-value	Accession no	Nearest phylogenetic neighbour	% similarity
22	0.0	AM177497.1	*Empedobacter brevis* strain LMG 4011^T partial 16S rRNA sequence	98%
		EF192137.1	*Flavobacterium* sp. ANU301 16S ribosomal RNA gene, partial sequence	98%
25	0.0	EU293154	*Empedobacter brevis* strain Y7D 16S ribosomal RNA gene, partial sequence	96%
		AM238680	*Wautersiella falsenii* genomovar 1 strain NF1182 partial 16S rRNA sequence	95%
27	0.0	AM177497	*Empedobacter brevis* strain LMG 4011^T partial 16S rRNA sequence	97%
		EF608515	Uncultured bacterium clone PCA-39 partial 16S rRNA sequence	97%

Table 4.13 16S rRNA-based identification of *Weeksella*-like isolates.

Isolate no	E-value	Accession no	Nearest phylogenetic neighbour	% similarity
28	0.0	EF204448	*Chryseobacterium b*ovis strain H15 partial 16S rRNA sequence	98%
		EF204446	*Chryseobacterium* bovis strain H9 partial 16S rRNA sequence	98%
30	0.0	EF204448.1	*Chryseobacterium bovis* strain H15 partial 16S rRNA sequence	98%
		EF204447.1	*Chryseobacterium bovis* strain H10 partial 16S rRNA sequence	98%
35	0.0	EF204448.1	*Chryseobacterium bovis* strain H15 partial 16S rRNA sequence	97%
		EF204447.1	*Chryseobacterium bovis* strain H10 partial 16S rRNA sequence	97%
40	0.0	AM982793.1	*Chryseobacterium* sp. CCUG 15260 partial 16S rRNA sequence	97%
		EF204450	*Chryseobacterium haifense* strain H38 partial 16S rRNA sequence	97%
43	0.0	EF204447.1	*Chryseobacterium bovis* strain H10 partial 16S rRNA sequence	97%
		EF204448.1	*Chryseobacterium bovis* strain H15 partial 16S rRNA sequence	97%
44	0.0	AJ271010.1	*Chryseobacterium* sp. UOF CM1396 partial 16S rRNA sequence	99%
		AY468479.1	*Chryseobacterium shigense* partial 16S rRNA sequence	99%
47	0.0	DQ279360	*Chryseobacterium* sp. TM3_8 partial 16S rRNA sequence	96%
		DQ279359	*Chryseobacterium* sp. TM11_4 partial 16S rRNA sequence	96%

Table 4.14 16S rRNA-based identification of the Unidentified SDS-PAGE isolates from the study of Jooste (1985) and Hugo (1997)

Isolate no	E-value	Accession no	Nearest phylogenetic neighbour	% similarity
50	0.0	AM232806.1	*Chryseobacterium ureilyticum* strain F-Fue-04IIIaaaa^T partial 16S rRNA sequence	99%
		AY345551.1	Bacterium H20 partial 16S rRNA sequence	99%
53	0.0	AJ583194.1	Uncultured CFB group bacterium clone S15D partial 16S rRNA sequence	99%
		AM232813.1	*Chryseobacterium indologenes* strain LMG 8337 partial 16S rRNA sequence	99%
57	0.0	AY468475	*Chryseobacterium* sp. LDVH 42/00 partial 16S rRNA sequence	95%
		AB245372	*Chryseobacterium ginsengisoli* strain G soil 183 partial 16S rRNA sequence	95%

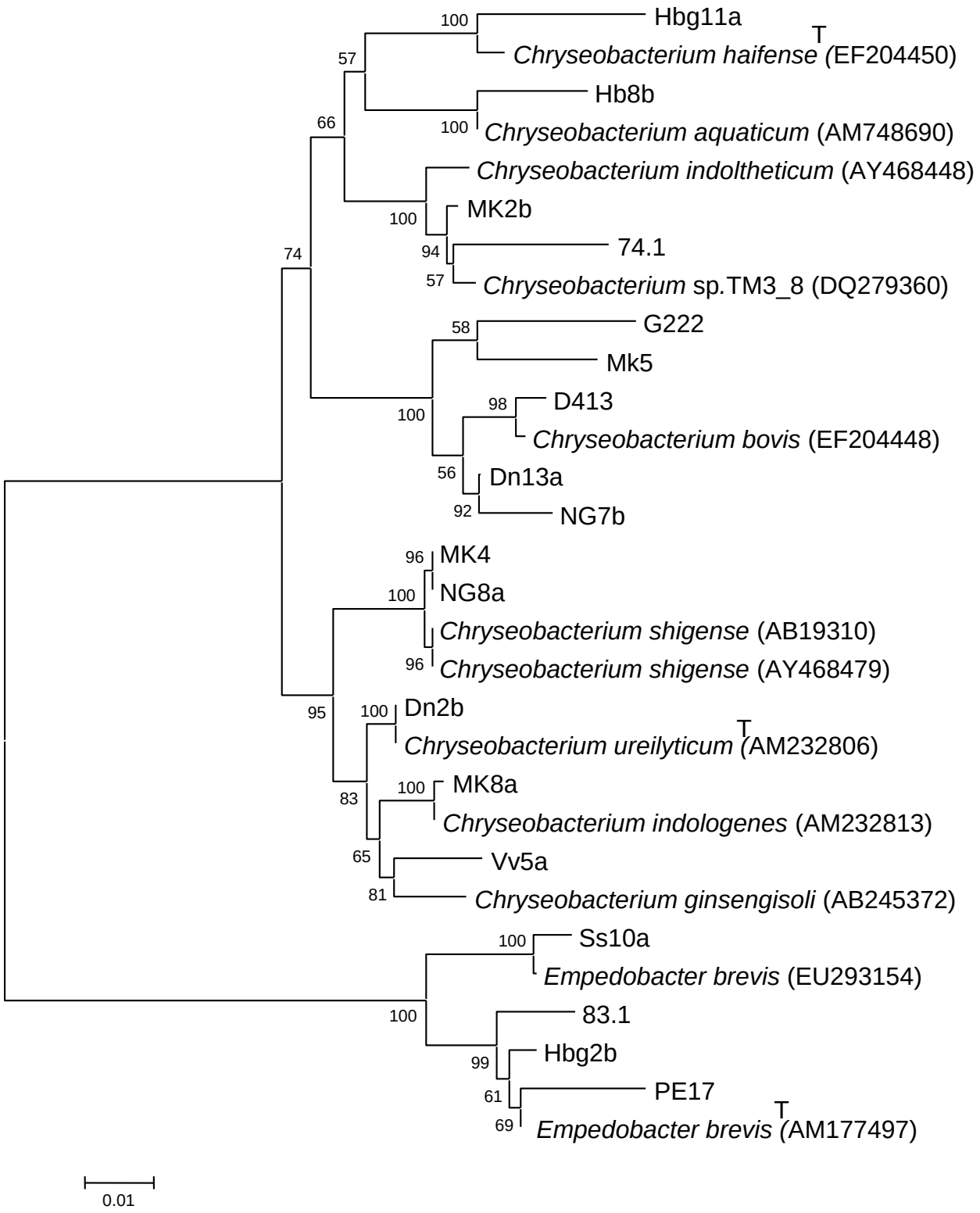

Fig.1. Neighbour joining phylogenetic tree showing relationships of 18 dairy isolates from this study and closely related species from the GenBank database. Bootstrap values are indicated at the nodes for 1000 replicates. The bar represents 10 substitutions per 100 nucleotides.

Isolate numbers 12 and 44 both formed a tight cluster with strains of *C. shigense* (AB19310) and (AY468479) respectively originally isolated from a lactic acid beverage by Shimomura *et al*. (2005). Isolate number 50 formed a tight cluster with *C. ureilyticum* with a sequence similarity of 99%, and isolate number 58 also formed a very tight cluster with *C. indologenes* with a sequence similarity of 99%. However, isolate number 57 showed a low similarity sequence of 96% to *C. ginsengisoli*. Sequence similarities in the range of 97% and 98% between *E. brevis* (EU293154) and isolate numbers 17, 22 and 27 formed a tight cluster suggesting a close phylogenetic relationship. Isolate number 25 formed a stable lineage with *E. brevis* (EU293154) isolated from soil contaminated with petroleum oil in China (GenBank: EU293154.1) characterized by a bootstrap value of 100%. But isolate numbers 17, 22 and 27 were phylogenetically closely related to *E. brevis* (AM177497) isolated from clinical sources such as blood, pus and ear discharge in Belgium (Kämpfer *et al*., 2006).

The phylogenetic tree was constructed to view the evolutionary distances between the members of the family *Flavobacteriaceae* isolated from milk and butter. All isolates depicted in the phylogenetic tree in Fig. 1, formed a close phylogenetic relationship.

Although there is absence of universal agreement about the % similarity required to assign a sequence to a particular species or genus, several cut-off similarity levels have been suggested ranging from 97% for the genus level to 99% for the species level (Drancourt *et al*., 2000; Stackebrandt *et al*., 2002). In general 16S rDNA similarities below 97% are indicative of novel species (Bosshard *et al*., 2003). Even though only partial sequences were obtained in this study it is likely that some of these isolates are indicative of new species. It is, therefore, suggested that isolate numbers 7 (*Chryseobacterium balustinum*-like), 25 (*Empedobacter*-like) and 57 (unidentified dairy isolate) be further investigated since they had % similarities below 97% (Tables 4.11, 4.12 and 4.15). It was found that the dairy isolates investigated using the 16S rRNA gene sequencing technique was significantly

identifiable at species level. The overall performance of the 16S rRNA gene sequencing method was good, because it was able to minimise the variable results obtained from the phenotypic methods.

4.1.3 Comparison of phenotypic and genotypic identification of dairy isolates

The comparison of the identification of 18 dairy isolates by the BIOLOG system (phenotypic) and the 16S rRNA method (genotypic) is given in Table 4.15. Most of the *C. balustinum*-like isolates were shown to belong to the *Chryseobacterium* genus using both methods. The BIOLOG system could, however, not identify the *Chryseobacterium* species to the same degree as the 16S rRNA method because of the limited number of *Chryseobacterium* species in the 2001 database. Both methods identified the three *Empedobacter*-like group isolates as *E. brevis*.

The results of the phenotypic results (Table 4.4) already indicated that the *Weeksella*-like isolates were a heterogeneous group of organisms. Neither method was able to place any of the isolates in the *Weeksella* genus. The reason for this was that the study of Jooste (1985) used phenotypic characteristics to place these isolates in a group that more closely reflected the characteristics of *Weeksella*. Interestingly, the only two butter isolates used for this part of the study, belonged in this group and were both identified as *C. bovis* by the 16S rRNA method. According to the latter method all 7 isolates evaluated belonged to *Chryseobacterium* species. The BIOLOG system, however, could only place three of these isolates in the *Chryseobacterium* genus and not in the same genera as the 16S rRNA method. Again, this could be attributed to the limited database of BIOLOG. This study, therefore, emphasized the importance of combining phenotypic and genotypic methods for better identification of organisms.

Table 4.15 Phenotypic and genotypic identification of dairy isolates. (*C*, *Chryseobacterium*; *E., Empedobacter*; -, no results).

Isolate no	Isolate code	BIOLOG	16S rRNA
7	Hb8b	*C. gleum/indologenes*	*C. aquaticum*
12	MK4	*C. gleum/indologenes*	*C. shigense*
13	MK5	*C. meningosepticum*	*C. bovis*
14	MK2b	-	*C. indoltheticum*
17	PE17	*C. indoltheticum*	*E. brevis*
22	Hbg2b	*E. brevis*	*E. brevis*
25	SS10a	CDC group ll-E subgroup A	*E. brevis*
27	83.1	*E. brevis*	*E. brevis*
28	D413	*Flavobacterium mizutaii*	*C. bovis*
30	Dn13a	*C. scophthalmum*	*C. bovis*
35	G222	*E. brevis*	*C. bovis*
40	Hbg11a	CDC group ll-E subgroup A	*C. haifense*
43	NG7b	-	*C. bovis*
44	NG8a	*C. gleum/indologenes*	*C. shigense*
47	74.1	*C. indoltheticum*	*Chryseobacterium* sp.
50	Dn2b	*E. brevis*	*C. ureilyticum*
53	MK8a	*C. gleum/indologenes*	*C. indologenes*
57	Vv5a	*C. gleum/indologenes*	*C. ginsengisoli*

Except for one isolate, all three isolates of the unidentified SDS-PAGE group were shown to belong to the *Chryseobacterium* genus by both methods. Isolate number 50 was, however, wrongly identified as *E. brevis* by the BIOLOG system. The reason for this is that the phenotypic characteristics of *Empedobacter* and *Chryseobacterium* are very close to each other. In fact,

both of these genera originally belonged to the *Flavobacterium* genus (Holmes *et al*., 1984a).

4.1.4 Summary of taxonomic findings based on phenotypic and genetic data of vegetable, soil and dairy isolates

This discussion will summarize the taxonomic findings based on the BIOLOG results of all the isolates (67) from vegetables and/or soil and dairy and are given in Table 4.16.

The eight isolates from vegetables (mushrooms and carrots) were identified as *Sphingobacterium* species with six isolates belonging to *S. mizutaii*, one isolate to *S. multivorum* and one isolate to *S. thalpophilum*. This implies that *Sphingobacterium* made out 11.9% of all the isolates studied. The two soil isolates belonged to the *Chryseobacterium* genus and although they were identified as *C. gleum/indologenes*, there is a possibility that these two isolates could belong to other or new species in this genus.

A total number of 23 isolates belonged to the genus *Chryseobacterium* in this study (34.3% of the total number of isolates in this study). Of these isolates 21 were from dairy sources and two were from the soil surrounding the lettuce plants (see section 4.1.1.). Sixteen of the *Chryseobacterium* isolates initially belonged to the *Chryseobacterium balustinum*-like and *Weeksella*-like groups from previous studies (Jooste, 1985; Hugo, 1997) and five belonged to the unidentified SDS-PAGE groups in the studies referred to.

A total of 14 strains (20.8% of the total number of isolates in this study) were found to be *Empedobacter brevis* and were previously isolated during the studies of Jooste (1985) and Hugo (1997) from dairy products. Of the 14 isolates 7/14, 5/14 and 2/14 were obtained from the *Empedobacter*-like, *Weeksella*-like- and the SDS-PAGE groups respectively.

Table 4.16 Summary of *Flavobacteriaceae* isolates identified using BIOLOG (2001) and 16S rRNA data. (n, number of isolates evaluated; %, percentage isolates of total; -, not performed).

Bacterial group/species	BIOLOG n=67	16S rRNA n=18	Total identified	% identified
Chryseobacterium spp.			23	34.3
C. gleum/indologenes	12	1		
C. aquaticum	0	1		
C. balustinum	4	0		
C. bovis	0	5		
C. ginsengisoli	0	1		
C. haifense	0	1		
C. indoltheticum	6	1		
C. scophthalmum	1	0		
C. shigense	0	2		
C. ureilyticum	0	1		
Chryseobacterium sp.	0	1		
Empedobacter brevis	14	4	14	20.8
Sphingobacterium spp.			8	11.9
S. mizutaii	6	-		
S. multivorum	1	-		
S. thalpophilum	1	-		
Elizabethkingia meningoseptica	6	0	6	9.0
CDC IIE (A) + (B)	4	0	4	6.0
Flavobacterium hydatis	1	0	1	1.5
Riemerella anatipestifer	1	0	1	1.5
Unidentified	10	0	10	15
Total			**67**	**100**

Six isolates (9% of the total number of isolates in this study) from dairy products were identified as *Elizabethkingia meningoseptica* and one originated in the unidentified SDS-PAGE group and five from the *Chryseobacterium balustinum*-like group from the studies of Jooste (1985) and Hugo (1997).

Four isolates (6% of the total number of isolates in this study) identified as CDC-group ll-E subgroup A and B respectively, were obtained from the *Empedobacter*-like and *Weeksella*-like groups in the studies of Jooste (1985) and Hugo (1997).

Flavobacterium hydatis and *Riemerella anatipestifer* (1.5% of the total number of isolates in this study) belonged to the original *Chryseobacterium balustinum*-like and *Weeksella*-like groups respectively.

Ten isolates (15% of the total number of isolates in this study) could not be identified by the BIOLOG phenotypic system and none of them underwent 16S rRNA sequencing.

Eighteen of sixty-seven isolates were identified using 16S rRNA sequencing; however the remaining 49 will be identified using the16S rRNA and other relevant genetic methods in the future. The identity of all 67 isolates will be verified using the polyphasic approach suggested by Bernardet *et al*. (2002).

4.2 Spoilage characteristics of flavobacterial isolates

The 28 flavobacterial isolates used in this part of the study, included 10 vegetable and soil isolates, 18 dairy isolates that were identified by the 16S rRNA method (14 *Chryseobacterium* and four *Empedobacter brevis* isolates) and two *Chryseobacterium* and eight *Sphingobacterium* isolates from soil and vegetables identified using the BIOLOG system.

The BIOLOG Microplate system is not only useful for identifying organisms, but can also be used to anticipate the possible spoilage characteristics since it contains a wide range of possible carbon sources (Mielmann, 2006). Instead of discussing the utilisation of each carbon source individually or one by one, the carbon sources that are most frequently utilized by bacteria can also be discussed in terms of so called chemical guilds (Preston-Mafham *et al*., 2002) of 10 carbohydrates, six carboxylic acids, five polymers, 13 amino acids and five miscellaneous carbon sources. The carbon sources that were most utilized by flavobacteria are presented in Tables 4.17a to 4.17d.

The presence of flavobacteria should not be discounted in food, because not only are they implicated in the food environment, but also cause spoilage. Their significance in dairy products is well documented and reports as early as that of Wolochow *et al*. (1942) provide evidence of their practical importance. They have the ability to spoil food because they produce thermostable enzymes such as proteases and lipases which play an important role in spoilage of dairy products. The spoilage characteristics of flavobacteria have been proved in previous studies using different techniques. Jooste *et al*. (1985) proved that flavobacteria played a proteolytic role in milk. Flavobacteria were grown on caseinate agar at different temperatures to test their ability to hydrolyse casein. It was concluded that flavobacteria was able to hydrolyse casein and this could cause practical problems in milk.

The food industry should maintain good hygienic practices to avoid contamination of food by flavobacteria. In previous studies, flavobacteria were isolated from South African fish species at the processing site and were considered potential active spoilers of the fish (Engelbrecht *et al*., 1996). During a more recent study, 36 *C. vrystaatense* isolates were obtained from raw chicken at different stages of processing at a chicken-processing plant in South Africa (de Beer *et al*., 2005). Flavobacteria have long been found to occur frequently in the dairy processing industry (Jooste *et al*., 1985; Welthagen and Jooste, 1992). They were introduced into raw milk due to poorly sanitized equipment and had adverse effects on milk and dairy products after processing.

The BIOLOG Microplate system is equally important in determining the possible spoilage characteristics of flavobacteria, because it consists of a wide range carbon sources which are most likely to be found in food. Therefore the BIOLOG Microplate system is indicative of which carbon sources are metabolised by the isolates and supposedly cause spoilage at 16h and 24h at any temperature.

4.2.1 *Chryseobacterium* **species**

The carbohydrates metabolized by the 16 *Chryseobacterium* isolates isolated from dairy products and soil is presented in Table 4.17a. α-D-glucose was the most utilized carbohydrate with 87.5% (14/16) *Chryseobacterium* isolates utilizing this carbohydrate. Both *Chryseobacterium gleum/indologenes* isolates from soil were able to utilize α-D-glucose. All the dairy *Chryseobacterium* isolates, except *C. ureilyticum* and *C. ginsengisoli*, were able to utilize α-D-glucose.

Table 4.17a. Carbohydrates utilized by the dairy, vegetable and soil isolates in this study. (V1-8, *Sphingobacterium*; **S9, S10,** *C. gleum/indologenes*; **53,** *C. indologenes*; **7,** *C. aquaticum*; **12, 44,** *C. shigense*; **13, 28, 30, 35, 43,** *C. bovis*; **14,** *C. indoltheticum*; **47,** *Chryseobacterium* sp. :**40,** *C. haifense*; **50,** *C. ureilyticum*; **57,** *C. ginsengisoli*; **17, 22, 25, 27,** *E. brevis***).**

Isolate number	α-D-Glucose	β-Methyl-D-glucoside	D-Arabinose	D-Cellobiose	D-Mannose	D-Psicose	D-Trehalose	Gentiobiose	Maltose	N-Acetyl-D-glucosamine
V1	+	+	+	+	+	-	+	+	+	-
V2	+	-	+	+	-	-	+	+	-	-
V3	+	-	-	+	-	-	+	+	-	+
V4	+	-	+	+	-	-	+	+	-	-
V5	-	+	-	+	-	+	-	+	-	-
V6	+	-	-	+	+	-	-	-	-	-
V7	+	+	-	+	-	-	-	+	-	-
V8	+	+	-	+	-	-	-	+	-	-
S9	+	-	-	-	-	-	-	+	-	-
S10	+	-	-	-	-	-	-	+	+	-
53	+	-	-	-	+	-	-	+	-	-
7	+	-	-	-	-	+	+	+	-	-
12	+	-	-	-	-	-	+	+	+	-
44	+	-	-	-	-	-	-	+	+	-
13	+	-	-	-	-	-	+	+	-	-
28	+	-	-	-	-	-	-	-	-	-
30	+	-	-	-	-	-	+	+	-	-
35	+	-	-	-	-	-	-	-	+	-
43	+	-	-	-	-	-	-	+	-	-
14	+	-	-	-	-	-	-	+	-	-
47	+	-	-	-	-	-	-	-	+	-
40	+	-	-	-	-	-	-	-	+	-
50	-	-	-	-	-	-	-	-	-	-
57	-	-	-	-	-	-	-	-	-	-
17	+	-	-	-	-	-	-	-	+	-
22	+	-	-	-	-	-	-	-	+	-
25	+	-	-	-	-	-	-	-	+	-
27	-	-	-	-	-	-	-	-	+	-

Gentiobiose was the second most utilized carbohydrate with 52.5% *Chryseobacterium* isolates utilizing this carbohydrate. The *C. gleum/indologenes* from soil*, C. aquaticum* and *C. shigense* isolates in this study were able to utilize gentiobiose, while the *C. bovis* isolates gave variable results.

Only 37.5% of *Chryseobacterium* isolates were able to utilize maltose. The *C. shigense* isolates were all able to utilize maltose while other *Chryseobacterium* isolates gave variable results with this carbohydrate.

D-Trehalose was utilized by only 25% of the *Chryseobacterium* isolates while no *Chryseobacterium* isolate was able to utilize any of β-methyl-D-glucoside, D-arabinose, D-cellobiose or N-acetyl-D-glucosamine.

Carbohydrates, if available, usually are preferred by microorganisms over other energy-yielding foods (Mountney and Gould, 1988). For utilisation, bacteria first need to break down complex carbohydrates such as starch into their constituent monosaccharides (Banwart, 1989). The random splitting of glycosidic bonds results in softening and liquefaction (Chesson, 1980).

Some of the metabolic products resulting from carbohydrate utilization (e.g. D-trehalose, gentiobiose) include organic acids, alcohols, CO_2, hydrogen and water (Banwart, 1989). Ayres *et al.* (1980) stated that the microbial fermentation of sugars can lead to souring and butyric spoilage defects.

Acetic acid was the carboxylic acid most utilized by *Chryseobacterium* isolates (75%). The *C. gleum/indologenes* from soil*, C. aquaticum, C. ureilyticum, C. ginsengisoli* isolates and one *C. shigense* isolate, one *C. indoltheticum* isolate and four *C. bovis* isolates from dairy products were able to utilize acetic acid (Table 4.17b). Only 18.8% *Chryseobacterium* isolates, namely *C. aquaticum and* two *C. shigense* isolates, utilized α-ketovaleric acid. One *C. gleum/indologenes* from soil and one *C. ginsengisoli*

Table 4.17b. Carboxylic acids utilized by the dairy, vegetable and soil isolates in this study. (V1-8, *Sphingobacterium*; **S9, S10,** *C. gleum/indologenes*; **53,** *C. indologenes*; **7,** *C. aquaticum*; **12, 44,** *C. shigense*; **13, 28, 30, 35, 43,** *C. bovis*; **14,** *C. indoltheticum*; **47,** *Chryseobacterium* sp. ; **40,** *C. haifense*; **50,** *C. ureilyticum*; **57,** *C. ginsengisoli*; **17, 22, 25, 27,** *E. brevis***).**

Isolate number	α-keto butyric acid	α-keto valeric acid	Acetic acid	Citric acid	Formic acid	Propionic acid
V1	-	-	-	-	-	-
V2	-	-	+	-	-	-
V3	-	-	-	-	-	-
V4	-	-	-	-	-	-
V5	-	-	+	-	-	-
V6	-	-	+	-	-	-
V7	-	-	-	-	-	-
V8	-	-	+	-	-	-
S9	+	-	+	-	-	-
S10	-	-	+	-	-	-
53	-	-	+	-	+	-
7	-	-	+	-	-	-
12	-	+	+	-	-	-
44	-	-	-	-	-	-
13	-	-	+	-	-	-
28	-	-	+	-	-	-
30	-	+	+	-	-	-
35	-	-	-	-	-	-
43	-	+	+	-	-	-
14	-	-	+	-	-	-
47	-	-	-	-	-	-
40	-	-	-	-	-	-
50	-	-	+	-	-	-
57	-	-	+	-	+	-
17	-	-	-	-	-	-
22	+	+	+	-	-	+
25	-	+	+	-	-	-
27	+	+	+	-	-	-

isolate 12.5% of *Chryseobacterium* isolates respectively, metabolized formic acid. No *Chryseobacterium* isolate could utilize citric acid or propionic acid.

The carboxylic acids (e.g. acetic acid) are organic compounds containing oxygen and are weak acids (Ebbing & Gammon, 1999). Many of these organic acids are oxidised by micro-organisms to carbohydrates, causing the medium to become more alkaline. Aerobically the organic acids may be oxidised completely to CO_2 and H_2O. Acids may also be oxidised to other, simpler acids or to products similar to those produced from sugars (Frazier, 1988). The production of acids may result in sour tastes and according to Coultate (1984); α-acids can lead to bitter tastes in foods.

The utilization of polymers by the *Chryseobacterium* isolates from soil and dairy is indicated in Table 4.17c. Dextrin was utilized by 56.3% while α-cyclodextrin was utilized by 37.5% of the *Chryseobacterium* isolates. The *C. gleum/indologenes* from soil, *C. aquaticum, C. shigense*, *C. indoltheticum, Chryseobacterium* sp. and three *C. bovis* isolates from dairy products utilized dextrin and α-cyclodextrin. Glycogen was utilized by 25% of the *Chryseobacterium* isolates, namely *C. aquaticum* and *C. bovis* as well as *C. gleum/indologenes* soil isolates. Tween 40 and Tween 80 were both utilized by one strain each of *C. indologenes, C. shigense* and *C. bovis*. The polymers include polysaccharides (Holding and Collee, 1971) and the breakdown of polysaccharides can cause changes in food texture (Ayres *et al*., 1980). Tween 40 and 80 provide suitable conditions for the establishment of lipase and other esterases (Harrigan and McCance, 1976), which can lead to spoilage of the final products during storage.

The amino acids utilized most by *Chryseobacterium* isolates are presented in Table 4.17d. L- Glutamic acid, glycyl-L-glutamic acid and glycyl-L-aspartic acid were utilized by 68.8%, 62.5% and 56.3% of *Chryseobacterium* isolates, respectively. L-Glutamic acid was utilized by *C.*

Table 4.17c. Polymer substrates utilized by the dairy, vegetable and soil isolates in this study. (V1-8, *Sphingobacterium*; **S9, S10,** *C. gleum/indologenes*; **53,** *C. indologenes*; **7,** *C. aquaticum*; **12, 44,** *C. shigense*; **13, 28, 30, 35, 43,** *C. bovis*; **14,** *C. indoltheticum*; **47,** *Chryseobacterium* sp. ; **40,** *C. haifense*; **50,** *C. ureilyticum*; **57,** *C. ginsengisoli*; **17, 22, 25, 27,** *E. brevis***).**

Isolate number	α-Cyclodextrin	Dextrin	Glycogen	Tween 40	Tween 80
V1	-	-	-	-	-
V2	-	-	-	-	-
V3	-	-	-	-	-
V4	-	-	-	-	-
V5	-	-	-	-	-
V6	-	-	-	-	-
V7	-	+	-	-	-
V8	-	-	-	-	-
S9	-	+	+	-	-
S10	-	+	+	-	-
53	-	-	-	+	+
7	+	+	+	-	-
12	+	+	-	+	+
44	-	-	-	-	-
13	+	+	-	+	+
28	-	-	-	-	-
30	-	-	-	-	-
35	+	+	-	-	-
43	+	+	+	-	-
14	-	+	-	-	-
47	+	+	-	-	-
40	-	-	-	-	-
50	-	-	-	-	-
57	-	-	-	-	-
17	+	+	-	-	-
22	+	+	+	+	+
25	+	+	-	+	+
27	-	-	-	-	-

Table 4.17d. Amino acid substrates utilized by the dairy, vegetable and soil isolates in this study. (V1-8, *Sphingobacterium*; **S9, S10,** *C. gleum/indologenes*; **53,** *C. indologenes*; **7,** *C. aquaticum*; **12, 44,** *C. shigense*; **13, 28, 30, 35, 43,** *C. bovis*; **14,** *C. indoltheticum*; **47,** *Chryseobacterium* sp. ; **40,** *C. haifense*; **50,** *C. ureilyticum*; **57,** *C. ginsengisoli*; **17, 22, 25, 27,** *E. brevis***).**

Isolate number	Glycyl-L-aspartic acid	Glycyl-L-glutamic acid	L-Alanine	L-alanyl-glycine	L-Asparagine	L-Aspartic acid	L-Glutamic acid	L-Leucine	L-Phenylalanine	L-Proline	L-Serine	L-Threonine
V1	-	+	-	-	+	-	-	-	-	-	-	-
V2	+	-	-	-	-	-	-	-	-	-	+	-
V3	+	-	-	-	-	-	-	-	-	-	-	-
V4	-	-	+	+	-	+	+	+	-	-	-	-
V5	-	-	-	-	-	-	-	-	-	-	-	-
V6	-	-	-	-	-	-	-	-	-	-	-	-
V7	-	-	-	-	-	+	+	-	-	-	-	-
V8	-	-	-	-	-	-	-	-	-	-	+	-
S9	+	-	+	-	+	-	-	+	-	-	-	-
S10	+	+	+	-	+	-	-	+	-	-	-	-
53	-	-	-	-	-	+	+	+	-	-	-	+
7	-	+	-	-	+	-	+	-	-	-	-	-
12	+	+	-	+	+	+	+	-	-	-	-	-
44	-	-	-	-	-	-	+	-	-	-	-	-
13	+	+	-	+	+	+	+	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-
30	-	+	-	-	-	-	+	-	-	-	-	-
35	+	+	-	-	-	-	-	-	-	-	-	-
43	+	+	-	-	-	-	+	-	-	-	-	-
14	+	+	-	-	+	-	+	-	-	-	+	+
47	-	+	-	-	-	-	+	-	-	-	-	-
40	+	+	-	-	-	-	+	-	-	-	-	-
50	-	-	-	-	-	-	+	-	-	-	-	-
57	+	-	+	-	-	-	-	+	-	-	-	-
17	+	+	-	-	-	-	-	-	-	-	-	-
22	+	+	+	+	+	+	+	-	-	-	-	-
25	+	+	-	-	-	-	+	-	-	-	-	-
27	+	+	-	+	+	+	+	-	-	-	-	-

aquaticum, one strain of *C. indologenes,* C*. shigense* isolates, three *C. bovis* isolates, *C. indoltheticum, C. haifense* and *C. ureilyticum*. Glycyl-L-glutamic acid was utilized by one *C. gleum/indologenes* soil isolate, *C. aquaticum*, *C. indoltheticum*, *C. haifense*, *C. shigense*, *Chryseobacterium* sp. and four *C. bovis* isolates.

L-aspartic acid was utilized by *C. aquaticum, C. haifense, C. ginsengisoli*, *C. gleum/indologenes* from soil and three *C. bovis* isolates. The *C. gleum/indologenes* isolates from soil and *C. ginsengisoli* were able to utilize L-alanine. L-alanyl-glycine was utilized by one *C. bovis* isolate and *C. shigense*. L-asparagine was utilized by *C. aquaticum*, *C. indoltheticum*, *C. shigense* and one *C. bovis* isolate. L-aspartic acid was utilized by *C. indologenes, C. shigense* and one *C. bovis* isolate. L-leucine was utilized by C. *gleum/indologenes* soil isolates, *C. indologenes* and *C. ginsengisoli*. L-serine was only utilized by *C. indoltheticum*, while L-threonine was utilized by *C. indologenes* and *C. indoltheticum*.

L-phenylalanine and L-proline could not be utilized by any of the *Chryseobacterium* isolates.

Anaerobic decomposition of amino acids may result in the production of obnoxious odours and the process is known as putrefaction. These foul-smelling, products include hydrogen, methyl and ethyl sulphides, mercaptans, ammonia, biogenic amines, indole, skatole and fatty acids (Frazier, 1988). According to Ayres *et al.* (1980), the incomplete metabolism of amino acids can result in putrescence. The formation of ammonia from amino acids can result in alkalinisation while the liberation of hydrogen sulphide (H_2S) from amino acids can result in sulphide spoilage. According to Coultate (1984) and Venter *et al.* (1999), bitterness is exclusively a characteristic of the hydrophobic L-amino acids (e.g. leucine). Off-odours and off-flavours described as metallic, unclean, bitter and putrid are produced by the breakdown of amino acids used as energy sources by the organism (Garbutt, 1997).

4.2.2 *Sphingobacterium* species

A total of eight *Sphingobacterium* isolates were evaluated for their spoilage characteristics. The utilization of carbohydrates by this genus is shown in Table 4.17a. All eight (100%) of the *Sphingobacterium* isolates could utilize D-cellobiose while seven of the eight *Sphingobacterium* isolates (87.5%) utilized α-D-glucose and gentiobiose, respectively. One (12.5%) *Sphingobacterium* isolate utilized D-psicose, maltose and *N*-acetyl-D-glucosamine. Three (37.5%) *Sphingobacterium* isolates utilized D-arabinose and two (25%) utilized D-mannose. Four isolates (50%) utilized β-methyl-D-glucoside and D-trehalose.

The *Sphingobacterium* isolates differed from the *Chryseobacterium* isolates mainly in the ability to utilize D-cellobiose and β-methyl-D-glucoside.

The only carboxylic acid utilized by the *Sphingobacterium* isolates was acetic acid (Table 4.17b), but only 50% of the isolates could do so. No other carboxylic acid could be utilized by the *Sphingobacterium* isolates. The *Chryseobacterium* isolates also mainly utilized this carboxylic acid.

Although mushrooms are a rich source of glycogen (Mattila *et al*., 2002), this polymer could not be used by any of the *Sphingobacterium* isolates (Table 4.17c). Only one (12.5%) of the *Sphingobacterium* isolates was able to utilize dextrin. This contrasts with the *Chryseobacterium* isolates, which were able to utilize a larger range of polymers.

Sphingobacterium isolates were not major amino acid utilizers in comparison with the *Chryseobacterium* isolates (Table 4.17d). One (12.5%) *Sphingobacterium* isolate each utilized glycyl-L-glutamic acid, L-alanine, L-alanyl-glycine, L-asparagine and L-leucine. Two isolates (25%) each were able to utilize glycyl-L-aspartic acid, L-aspartic acid, L-glutamic acid and L-serine. As in the case with *Chryseobacterium, Sphingobacterium* was also not able to utilize L-phenylalanine and L-proline.

Sphingobacterium and *Empedobacter* cultures are known for the production of a strong “fruity” odour (Yabuuchi *et al.*, 1983; 1990). Fruity odours arise from the degradation of the amino acids glycine, leucine and serine to form lower fatty acids (Engelbrecht *et al.*, 1996). The breakdown of vegetable components by spoilage bacteria may give rise to a soft, mushy consistency, a bad odour and a water-soaked appearance (Jay, 1996). Vitamins and minerals present in plant foods are also used by spoilage bacteria, resulting in adverse changes to the appearance and organoleptic quality of food (Banwart, 1981).

4.2.3 *Empedobacter* isolates

Table 4.17a indicates the carbohydrates utilized by *Empedobacter brevis* isolates. Carbohydrates are usually preferred by microorganisms to other energy-yielding carbon sources (Dainty, 1996). The only two carbohydrates that could be utilized by *Empedobacter* isolates were α-D-glucose and maltose. As with *Chryseobacterium* and *Sphingobacterium*, α-D-glucose was one of the major utilized carbohydrates and 75% of the *Empedobacter* isolates were able to use this monosaccharide. The *Empedobacter* isolates (100%), however, differed from the other two genera in this study in utilizing maltose.

The carboxylic acids utilized by four *Empedobacter* isolates are shown in Table 4.17b. α-Keto valeric acid and acetic acid were utilized by 75% of the *Empedobacter* isolates, while α-keto butyric acid was utilized by 50%.

The *Empedobacter* isolates differed from *Sphingobacterium* and *Chryseobacterium* isolates in utilizing α-keto butyric acid and α-keto valeric acid. As with the *Sphingobacterium* and *Chryseobacterium* isolates, *Empedobacter* isolates were also not able to utilize citric acid. It was discovered that the metabolism of carboxylic acids is essential for the production of flavour in cheese making (Urbach, 1997).

The utilization of polymers by the four *Empedobacter* isolates is shown in Table 4.17c. Three isolates (75%) were able to utilize α-cyclodextrin and dextrin. Only one isolate (25%) had the ability to utilize glycogen. Tween 40 and Tween 80 were metabolized by two isolates (50%) each.

Empedobacter and *Chryseobacterium* were both able to utilize polymers in this study, which were in contrast to *Sphingobacterium*, which were unable to utilize polymers.

Glycyl-L-aspartic acid and glycyl-L-glutamic acid were the major amino acids utilized by the four (100%) *Empedobacter* isolates (Table 7.17d). L-glutamic acid was utilized by 75% of the isolates. L-aspartic acid, L-asparagine and L-alanyl-glycine were all utilized by 50% of the isolates. The *Empedobacter* isolates were, however, unable to utilize L-leucine, L-proline, L-serine and L-threonine. Studies on the activities of flavobacteria (Cousin, 1982; Jooste, 1985) have indicated that flavobacteria produce pasteurisation resistant, extracellular enzymes and that they may contribute to the psychrotrophic spoilage of milk and dairy products. Flavobacteria were found to be highly proteolytic in this regard and the hydrolysis of proteins to peptones, polypeptides and amino acids is caused by proteolytic enzymes. Free amino acids often results in putrid off-odours associated with lower molecular-weight degradation products such as ammonia, amines and sulphides (Frank, 1997).

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Introduction

The taxonomy of the flavobacteria was in disarray for many years before the inception of the family *Flavobacteriaceae* suggested by Jooste (1985; Reichenbach, 1992). Currently, 76 genera have been included in this family. Although some genera were grouped early on based on common habitats and phenotypic traits, some of them were included in the family only after investigation using molecular methods (Bernardet and Nakagawa, 2003). Of the 76 genera currently known in this family, only 10 genera are associated with food (Hugo and Jooste, in press).

Flavobacteria have long been known for their proteolytic activity, causing spoilage of food, especially those rich in proteins such as milk and meat (Jooste and Britz, 1986; Venter *et al.*, 1999). The role and significance of the flavobacteria in these foods as well as in vegetables and soil and their proven and potential significance as food spoilage bacteria have not been studied in as much detail as the taxonomy and nomenclature of this family.

The aims of this project were, firstly, to investigate the identity of flavobacteria isolated from vegetables and the soil surrounding the vegetables, by using the BIOLOG system. This system was also used to identify possible spoilage characteristics of the identified isolates. Secondly, dairy isolates from the culture collection of the Free State that could previously not be identified to genus and/or species level, were subjected to BIOLOG identification as well as to 16S rRNA sequencing. Again, some identified isolates were used to determine possible spoilage characteristics using the BIOLOG system.

5.2 Identification of the vegetable and soil isolates

A total of 601 Gram-negative yellow pigmented rods were recovered from different vegetables and soils in the Free State province of South Africa as indicated in Table 3.1 in Chapter 3. The aim of this part of the study was to determine to what extent flavobacteria occur on vegetables and, if present, which components they degrade to bring about spoilage. All isolates were subcultured until purity was obtained for further analysis. The isolates underwent phenotypic tests proposed by Bernardet *et al*. (2002) and ten isolates phenotypically were found that closely resembled members of the family *Flavobacteriaceae*, particularly the genus *Chryseobacterium*.

The 10 isolates were subjected to the BIOLOG Microplate system for identification. The BIOLOG Microplate system tests the ability of a microorganism to utilize or oxidize compounds from a preselected panel of different carbon sources, giving identification of the organism after 16 and 24h of incubation. Eight isolates from mushrooms and carrots belonged to the genus *Sphingobacterium* in the family *Sphingobacteriaceae* (Steyn *et al*., 1998), whereas two isolates from the soil surrounding the lettuce belonged to the genus *Chryseobacterium* in the family *Flavobacteriaceae* as indicated in Table 4.2b. The reason for *Sphingobacterium* being isolated in this study is that its phenotypic characteristics closely resemble those of the *Flavobacteriaceae* family. In fact, this genus was formerly part of the *Flavobacteriaceae* family (Yabuuchi *et al*., 1983).

The 16S rRNA sequences of the 10 vegetable and soil isolates unfortunately were short and of poor quantity and no results could, therefore, be obtained. Presumably some DNA was lost after cleaning the PCR product using the Zymo Research DNA Clean and Concentrator for these samples. The quantity of the DNA template for sequencing is crucial and is a common problem when sequencing the PCR product. Too little DNA template reduces the signal

length and peak height, and this was detected with these samples (Clarridge, 2004).

5.3 Identification of the dairy isolates

Fifty seven dairy isolates obtained from milk and butter were used from previous studies and grouped as *C. balustinum*-like, *Empedobacter*-like, *Weeksella*-like (Jooste, 1985) and an unidentified SDS-PAGE group (Hugo *et al*., 1997). These isolates were subjected to phenotypic tests, BIOLOG and 16S rRNA sequencing for identification.

5.3.1. Phenotypic identification

With the phenotypic identification, the *C. balustinum*-like group had the overall characteristics of the genus *Chryseobacterium* as shown in Table 4.3. One of the characteristics of *Chryseobacterium*, according to Bernardet *et al*. (2002), is that all *Chryseobacterium* species except *C. proteolyticum* are resistant to penicillin G. This was indeed so for the isolates in this group. The *Empedobacter*-like and *Weeksella*-like dairy isolates did not warrant inclusion into the genera *Empedobacter* and *Weeksella* because they were phenotypically heterogeneous as shown in (Tables 4.4 and 4.5) respectively. It became evident that the unidentified SDS-PAGE group (Table 4.6) phenotypically closely resembled the genus *Chryseobacterium*.

5.3.2. BIOLOG identification

With BIOLOG identification, 13 of the 19 isolates of the *C. balustinum*-group were identified as belonging to *Chryseobacterium* although only two of the 19 isolates were identified as *C. balustinum* (Table 4.7). Seven of the eight isolates in the *Empedobacter*-group were identified by the BIOLOG system as *Empedobacter brevis* (Table 4.8), which is in accordance with the study by

Jooste (1985). None of the 20 isolates in the *Weeksella*-like group could be identified as *Weeksella*. Isolates in this group were identified as belonging to the genera *Chryseobacterium*, *Empedobacter*, *Riemerella* and the rest belonged to CDC group IIE subgroup A as presented in Table 4.9. The majority (6/10) of the isolates in the unidentified SDS-PAGE group belonged to the genus *Chryseobacterium*; two isolates belonged to the genus *Empedobacter*, while two isolates could not be identified with this technique (Table 4.10).

5.3.3. 16S rRNA sequencing identification

This technique was applied to 18 isolates; sixteen from milk and two from butter. Isolates were obtained from the *C. balustinum*-like, *Empedobacter*-like, *Weeksella*-like and the unidentified SDS-PAGE group. This technique was found to be a powerful tool to trace phylogenetic relationships between bacteria and to identify bacteria from various sources (Macrae, 2000).

From the *C. balustinum*-like group, five isolates were evaluated. Although all belonged to the *Chryseobacterium* genus, not one belonged to *C. balustinum* but to *C. shigense, C. indoltheticum, C. bovis, C. aquaticum* and *Empedobacter brevis* (Table 4.11). All three isolates from the *Empedobacter*-like group identified with this method were closely related to *E. brevis* with % similarities between 96%-98% as indicated in Table 4.12. The *Weeksella*-like group was the biggest dairy group in this study, but only seven of the 20 isolates were analyzed using this technique. All the isolates were related to the genus *Chryseobacterium*. Four isolates were closely related to *C. bovis* with different % similarities, two other *Weeksella-like* isolates belonged to different *Chryseobacterium* spp. and one isolate was only identified to the genus level (Table 4.13). The three isolates from the unidentified SDS-PAGE group were identified as *C. ureilyticum, C. indologenes* and *C. ginsengisoli* respectively (Table 4.14).

All of the dairy isolates analyzed with this method belonged to the family *Flavobacteriaceae*, and most of the isolates belonged to the genus *Chryseobacterium*. Some of the dairy isolates were closely related to *Chryseobacterium* species from the dairy environment, such as *C. bovis* and *C. haifense* which were both isolated from raw cow's milk during a study of the diversity of psychrotolerant bacteria in raw milk in Israel (Hantsis-Zacharov and Halpern, 2007; Hantsis-Zacharov *et al*., 2008) and *C. indologenes* which was also isolated from the dairy environment (Hugo and Jooste, 1997; Hugo *et al*., 1999). Other dairy isolates were generally related to different *Chryseobacterium* spp. isolated from the food environment such as *C. ureilyticum* isolated from the beer-bottling plants located in breweries in Germany (Hertzog *et al*., 2008) and *C. shigense* from a lactic acid beverage in Japan (Shimomura *et al*., 2005).

5.3.4. Comparison of phenotypic (BIOLOG) and genotypic (16S rRNA sequencing) identification

The comparison of the identification of 18 dairy isolates by the BIOLOG system (phenotypic) and the 16S rRNA method (genotypic) is given in Table 4.15. Most of the *C. balustinum*-like isolates were shown to belong to the *Chryseobacterium* genus using both methods, but not one isolate was identified as *C. balustinum*. The BIOLOG system could, however, not identify the *Chryseobacterium* species to the same degree as the 16S rRNA method because of the limited amount of *Chryseobacterium* species in the 2001 database. Both methods correctly identified the three *Empedobacter*-like group isolates as *E. brevis*.

Phenotypic data (Table 4.4) had already indicated that the *Weeksella*-like isolates were a heterogeneous group of organisms. Both methods, however, were unable to place any of the isolates in the *Weeksella* genus. The reason for this was that the study of Jooste (1985) only used phenotypic characteristics to place these isolates in a group that more closely resembled the characteristics of

Weeksella. According to the 16S rRNA method all 7 isolates evaluated belonged to *Chryseobacterium* species. Interestingly, both butter isolates (28, 35) were identified as *C. bovis*. The BIOLOG system, however, could only place three of these isolates in the *Chryseobacterium* genus and not in the same genera as the 16S rRNA method. Again, this could be attributed to the limited 2001 database of BIOLOG. This study, therefore, emphasized the value of using phenotypic and genotypic methods in combination for the more effective practical identification of aerobic, Gram-negative bacteria.

Except for one isolate, all three isolates of the unidentified SDS-PAGE group belonged to the *Chryseobacterium* genus using both methods. Isolate number 50 was, however, wrongly identified as *E. brevis* with the BIOLOG system. The reason for this is that the phenotypic characteristics of *Empedobacter* and *Chryseobacterium* are very similar to each other. In fact, *Empedobacter* was originally known as *Flavobacterium breve* which had previously, erroneously been grouped with other chryseobacteria as *Flavobacterium* species (Holmes *et al*., 1984a).

The BIOLOG system is a phenotypic method and requires verification. Phenotypic profiles including enzymatic and/or metabolic activities are crucial when investigating bacterial physiology, but these characteristics are not static and can change with stress such changes in temperature, nutrient availability, exposure to different media and periods of incubation during the research. This method is affected by a number of parameters, amongst them the growth rate of isolates. If the organisms grow slowly and do not utilise more than three carbon sources on the plate after 16 and 24h of incubation, then it cannot be identified. Identification sometimes is inaccurate because the 2001 database currently in use cannot be updated with newer or more recent bacterial names.

Although not perfect, genotypic identification of microorganisms by 16S rRNA gene sequencing has emerged as a more objective, accurate, and reliable

method for bacterial identification, with the added capability of defining taxonomical relationships among bacteria (Fredricks and Relman, 1996). The part of the DNA now commonly used for taxonomic purposes for bacteria is the 16S rRNA gene because it is highly conserved and universal in bacteria (Clarridge, 2004).

5.4 Summary of taxonomic findings based on phenotypic data of vegetable, soil and dairy isolates

This part of this study was aimed at summarizing the taxonomic findings based on the BIOLOG results of all the isolates (67) from vegetables and/or soil and dairy and are given in Table 4.16.

From vegetables (mushrooms and carrots), 11.9% of the isolates in this study belonged to the *Sphingobacterium* genus. The dairy and soil isolates was presented by *Chryseobacterium* species (34.3%), *Empedobacter brevis* (20.8%), *Elizabethkingia meningoseptica* (9%), CDC-group II-E subgroups A and B (6%), *Flavobacterium hydatis* (1.5%) and *Riemerella anatipestifer* (1.5%). Fifteen percent of the isolates in this study could not be identified with the BIOLOG phenotypic system.

5.5 Spoilage characteristics of vegetable/soil and dairy isolates

The BIOLOG system was also used to screen which carbon sources were utilized by the isolates in this study in order to predict possible spoilage characteristics of the organisms. The 10 vegetable isolates subjected to BIOLOG identification and the 18 isolates identified by the 16S rRNA method were subjected to this method. The most widely utilized carbohydrate by all the isolates was glucose which is the initial carbon source supporting growth of all the major types of bacteria and only when this carbon source is depleted, do

other substrates begin to be metabolised (Dainty, 1996). Gentiobiose and maltose were the second most widely used carbohydrates by the dairy isolates (8/18 and 7/18 isolates respectively); while both of the *Chryseobacterium* isolates from the vegetables were able to utilize gentiobiose. According to Banwart (1989) some of the metabolic products resulting from D-trehalose and gentiobiose utilization, include organic acids, alcohols, CO_2 and hydrogen, which may contribute to spoilage defects in food products.

Most of the dairy isolates (13/18) and both of the vegetable *Chryseobacterium* isolates utilized acetic acid as a carboxylic acid. The carboxylic acids (e.g. acetic acid) are organic compounds containing oxygen and are weak acids (Ebbing & Gammon, 1999). Many of these organic acids are oxidised by micro-organisms to carbohydrates, causing the medium to become more alkaline. Aerobically the organic acids may be oxidised completely to CO_2 and H_2O. The metabolism of carboxylic acids such as acetic, propionic, and formic acid by micro-organisms results in the coagulation of milk accompanied by sour and bitterness in milk (Urbach, 1997).

Glycyl-L-aspartic acid (11/18), glycyl-L-glutamic acid (13/18) and L-glutamic acid (14/18) were the amino acids most widely utilized by the dairy isolates while glycyl-L-aspartic acid, L-alanine L-asparagine and L-leucine were utilized by both vegetable *Chryseobacterium* isolates. The oxidation/reduction of amino acids results in organic acids, ammonia and carbon dioxide. For example, the break down of glutamic acid yields acetic acid, butyric acid, carbon dioxide (CO_2), ammonia and hydrogen. Butyric acid and ammonia impart obnoxious (rancid) odours to foods (Banwart, 1989). According to Ayres *et al.* (1980), the incomplete metabolism of amino acids can result in putrescence, while the liberation of hydrogen sulphide (H_2S) from amino acids can result in a sulphide type of spoilage. A sensory analysis study of protein hydrolysates has shown that bitterness due to the resulting free amino acids, is exclusively a characteristic of the hydrophobic L-amino acids (e.g. leucine) (Coultate, 1984). The major cause

of bitterness in milk and milk products is the formation of bitter peptides due to the action of proteinases on proteins such as the caseins in milk (Huis in't Veld, 1996).

Dextrin was the mostly utilized polymer by both *Chryseobacterium* vegetable isolates as well as 10/18 dairy isolates. Polymers include polysaccharides (Holding and Collee, 1971). The microbial production of polysaccharides (e.g. dextrins) from various disaccharides, present in food, can form unpleasant slime in and on food, causing the food to be both unpalatable and unacceptable to the consumer (Ayres *et al*., 1980). Although lipase activity has been reported for most psychotrophs (most *Flavobacteriaceae* genera from food are psychrotrophic) isolated from milk and milk products, only one *C. shigense* strain, one *C. bovis* strain, *C. indologenes* and two *E. brevis* strains were able to degrade Tween 60 and 80, which is indicative of possible lipolytic activity.

5.6 Recommendations for future research

- Since the identity of the two *Chryseobacterium* vegetable isolates are still not known, a polyphasic approach suggested by Bernardet *et al.* (2002) should be used to identify these two strains to species level. This should prove whether they belong to existing species or should be proposed as new species.
- Verification of the identity of the 18 dairy isolates by using the same polyphasic approach suggested by Bernardet *et al.* (2002).
- Investigation of spoilage characteristics of the same species on different food types.

CHAPTER 6

REFERENCES

Alain, K., Intertaglia, L., Catala, P., and Lebaron, P. (2008). *Eudoraea adriatica* gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **58**, 2275-2281.

Allen, T. D., Lawson, P. A., Collins, M. D., Falsen, E., and Tanner, R. S. (2006). *Cloacibacterium normanense* gen. nov., sp. nov., a novel bacterium in the family *Flavobacteriaceae* isolated from municipal wastewater. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1311-1316.

Asker, D., Beppu, T., and Ueda, K. (2007). *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family *Flavobacteriaceae*, isolated from seawater off Enoshima Island, Japan. *International Journal of Systematic and Evolutionary Microbiology* **57**, 837-843.

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

Babic, I., Roy, S., Watada, A. E., and Wergin, W. P. (1996). Changes in microbial populations on fresh-cut spinach. *International Journal of Food Microbiology* **31**, 107-119.

Bae, S. S., Kwon, K. K., Yang, S. H., Lee, H.-S., Kim, S.-J., and Lee, J.-H. (2007). *Flagellimonas eckloniae* gen. nov., sp. nov., a mesophilic marine bacterium of the family *Flavobacteriaceae*, isolated from the rhizosphere of *Ecklonia kurume*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 1050-1054.

Banwart, G. J. (1981). *Basic Food Microbiology*. AVI Publishing Company, Inc., Westport, CT.

Banwart, G. J. (1989). *Basic Food Microbiology*, 2nd edn. Van Nostrand Reinhold, New York.

Barbeyron, T., L'Haridon, S., Corre, E., Kloareg, B., and Potin, P. (2001). *Zobellia galactonovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and classification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **51**, 985–997.

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

Beattie, G. A. (2006). Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: *Plant-Associated Bacteria*, ed. S.S. Gnanamanickam. Springer, The Netherlands, pp. 1-56.

Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, R. S., Hammer, B. W., and Huntoon, F. M. (1923). *Bergey's Manual of Determinative Bacteriology*, 1st edn., Williams and Wilkins, Baltimore, pp. 97-117.

Bernardet, J.-F. (in press). Family I. *Flavobacteriaceae*. Reichenbach 1992, 327VP (Effective publication: Reichenbach 1989, 2013) emend. Bernardet, Segers, Vancanneyt, Berthe, Kersters, and Vandamme, 1996, 145VP. In: *Bergey's Manual of Systematic Bacteriology.*

Bernardet, J.-F., and Bowman, J.P. (2006). Chapter 6.4 The genus *Flavobacterium*. In: *The Prokaryotes* Vol. 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria, 3rd edn., eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. Springer Verlag, New York, pp. 481-531.

Bernardet, J.-F., and Nakagawa, Y. (2006). An introduction to the family *Flavobacteriaceae*. In: *The Prokaryotes* Vol 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria, 3rd edn., eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. Springer Verlag, New York, pp. 455-480.

Bernardet, J.-F., Hugo, C., and Bruun, B. (2006). The genera *Chryseobacterium* and *Elizabethkingia*. In: *The Prokaryotes* Vol. 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria, 3rd edn., eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. Springer Verlag, New York, pp. 638-676.

Bernardet, J.-F., Vancanneyt, M., Matte-Tailliez, O., Grisez, L., Taillez, P., Bizet, C., Nowakowski, M., Kerouault, B., and Swings, J. (2005). Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals. *Systematic and Applied Microbiology* **28**, 640-660.

Bernardet, J.-F., Nakagawa, Y., and Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emendation of the family. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1049-1070.

Bernardet, J.-F., and Nakagawa, Y. (2003). An introduction to the family *Flavobacteriaceae.* In: *The Prokaryotes, An Evolving Electronic Resource for the Microbiological Community*, release 3.15. eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. Springer Verlag, New York. http://link.springer.ny.com.service/books/10125/

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K., and 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). *International Journal of Systematic Bacteriology* **46**, 128–148.

Bernardet, J.-F., and Grimont, P. A. D. (1989). Deoxiribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida and Masumura, 1886. *International Journal of Systematic Bacteriology* **39**, 346-354.

Bolton, H., Fredrickson, J. K., and Elliott, L. F. (1993). Microbial ecology of the rhizosphere. In: *Soil Microbial Ecology*, ed. F. B. Metting. Marcel Dekker, New York, pp. 27-63.

Bosshard, P. P., Abels, S., Zbinden, R., Bottger, E.C., and Altwegg, M. (2003). Ribosomal DNA sequencing for identification of aerobic Gram-positive rods in the clinical laboratory (an 18-month evaluation). *Journal of Clinical Microbiology* **41**, 4134-4140.

Botha, W. C., Jooste, P. J., and Britz, T. J. (1989). The taxonomic relationship of certain environmental flavobacteria to the genus *Weeksella*. *Journal of Applied Bacteriology* **67**, 551-559.

Botha, W. C., Jooste, P. J., and Hugo, C. J. (1998a). The incidence of *Weeksella*- and *Bergeyella*-like bacteria in the food environment. *Journal of Applied Microbiology* **84**, 349-356.

Botha, W. C., Jooste, P. J., and Hugo, C. J. (1998b). Taxonomic interrelationship of *Weeksella*- and *Bergeyella*-like strains from dairy sources using electrophoretic protein profiles, DNA base composition and non-polar fatty acid composition. *Food Microbiology* **15**, 479-489.

Bowman, J. P., and Nichols, D. S. (2002). *Aequorivita* gen. nov., a member of the family *Flavobacteriaceae* isolated from terrestrial and marine Antarctic habitats. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1533-1541.

Bowman, J.P., and Nichols, D.S. (2005). Novel members of the family *Flavobacteriaceae* from Antarctic maritime habitats including *Subsaximicrobium wynnwilliamsii* gen. nov., sp. nov., *Subsaximicrobium saxinquilinus* sp. nov., *Subsaxibacter broadyi* gen. nov., sp. nov., *Lacinutrix copepodicola* gen. nov., sp. nov., and novel species of the genera *Bizionia*, *Gelidibacter* and *Gillisia*. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1471-1486

Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S., and McMeekin, T. A. (1997a). *Psychroserpens burtonensis* gen. nov., sp. nov., and *Gelidibacter algens* from Antarctic lacustrine and sea ice habitats. *Applied and Environmental Microbiology* **63**, 3068-3078.

Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S., and McMeekin, T. A. (1997b). Diversity and association of psychrophilic bacteria in Antartic sea ice. *Applied and Environmental Microbiology* **63**, 3068-3078.

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

Brambilla, E., Päuker, O., Cousin, S., Steiner, U., Reimer, A., and Stackebrandt, E. (2007). High phylogenetic diversity of *Flavobacterium* spp. isolated from a hardwater creek, Harz Mountains, Germany. *Organisms Diversity and Evolution* **7**, 145-154.

Brummer, I. H. M., Fehr, W., and Wagner-Döbler, I. (2000). Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. *Applied and Environmental Microbiology* **66**, 3078-3082.

Bruns, A., Rohde, M., and Berthe-Corti, L. (2001). *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. *International Journal of Systematic and Evolutionary Microbiology* **51,** 1997-2006.

Bryan, F. L. (1980). Poultry and poultry meat products. In: *Microbial Ecology of Foods,* eds. J. H. Silliker, R. P. Elliot, A. C. Baird-Parker, F. L. Bryan, J. H. B. Christian, D. S. Clark, J. C. Olsen Jr., and T. A. Roberts. Academic Press, New York, pp. 410-469.

Callon, C., Duthoit, F., Delbès, C., Ferrand, M., Le Frileaux, Y., De Crèmoux, R., and Montel, M.-C. (2007). Stability of microbial communities in goat milk during a lactation year: molecular approaches. *Systematic and Applied Microbiology* **30**, 547-560.

Campbell, L. L., and Williams, O. B. (1951). A study of chitin-decomposing microorganisms of marine origin. *Journal of General Microbiology* **5**, 879-905.

Campbell, R., and Greaves, M. P. (1990). Anatomy and community structure of the rhizosphere. In: *The Rhizosphere*, ed. J. M. Lynch. John Wiley & Sons Chichester, England, pp. 11-34.

Campbell, S., Harada, R. M., and Li, Q. X. (2008). *Chryseobacterium arothri* sp. nov., isolated from the kidneys of a puffer fish. *International Journal of Systematic and Evolutionary Microbiology* **58**, 290-293.

Chen, Y. G., Cui, X. L., Zhang, Y. Q., Li, W. J., Wang, Y. X., Kim, C. J., Lim, J. M., Xu, L. H., and Jiang, C. L. (2008). *Salinimicrobium terrae* sp. nov., isolated from saline soil, and emended description of the genus

Salinimicrobium. *International Journal of Systematic and Evolutionary Microbiology* **58**, 2501-2504.

Chesson, A. (1980). Maceration in relation to the post-harvest handling and processing of plant material. *Journal of Applied Bacteriology* **48,** 1-45.

Cho, J. C., and Giovannoni, S. J. (2003). *Croceibacter atlanticus* gen. nov., sp. nov., a novel marine bacterium in the family *Flavobacteriaceae*. *Systematic and Applied Microbiology* **26**, 76-83.

Cho, J. C., and Giovannoni, S. J. (2004). *Robiginitalea biformata* gen. nov., sp. nov., a novel marina bacterium in the family *Flavobacteriaceae* with a higher G + C content. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1101-1106.

Choi, D. H., and Cho, B. C. (2006). *Lutibacter litoralis* gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae* isolated from tidal flat sediment. *International Journal of Systematic and Evolutionary Microbiology* **56**, 771-776.

Chourasia, H. K. (1995). Kernel infection and aflatoxin production in peanut (*Arachis hypgaea* L.) by *Aspergillus flavus* in presence of geocarposhere bacteria. *International Journal of Food Science and Technology* **32**, 459-464.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. *Clinical Microbiological Reviews* **17**, 840-862.

Cottrell, M. T., and Kirchman, D. L. (2000). Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence *in situ* hybridization. *Applied and Environmental Microbiology* **66**, 5116-5122.

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

Cousin, M. A. (1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. *Journal of Food Protection* **45**, 172-207.

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

Daud, H. B., McMeekin, T. A., and Thomas, C. J. (1979). Spoilage association of chicken skin. *Applied and Environmental Microbiology* **37**, 399-401.

Dainty, R. H. (1996). Chemical/biochemical detection of spoilage. *International Journal of Food Microbiology* **33**, 19-33.

de Beer, H., Hugo, C. J., Jooste, P. J., Willems, A., Vancanneyt, M., Coenye, T., and Vandamme, P. A. R. (2005). *Chryseobacterium vrystaatense* sp. nov., isolated from raw chicken in a chicken-processing plant. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2149-2153.

de Beer, H., Hugo, C. J., Jooste, P. J., Vancanneyt, M., Coenye, T. and Vandamme, P. (2006). *Chryseobacterium piscium* sp. nov., isolated from fish of the South Atlantic Ocean off South Africa. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1317-1322.

Dobson, S. J., Colwell, R. R., McMeekin, T. A., and Franzmann, P. D. (1993). Direct sequencing chain reaction-amplified 16S rRNA gene of *Flavobacterium gondwanense* sp. nov. and *Flavobacterium salegens* sp. nov., two new species from a hypersaline Antartic lake. *Journal of Applied Bacteriology* **43**, 77-83.

Domenech, J., Reddy, M. S., Kloepper, J. W., Ramos, B., and Gutierrez-Manero, J. (2006). Combined application of the biological product LS213 with

Bacillus, Pseudomonas or *Chryseobacterium* for growth promotion and biological control of soil-borne disease in pepper and tomato. *BioControl* **51**, 245-258.

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

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

Eilers, H. J., Pernthaler, J ., Glöckner, F. O., and Amman, R. (2000). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology* **66**, 77-83.

Einen, J., and Øvreas, L. (2006). *Flaviramulus basaltis* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from seafloor basalt. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2455-2461.

Elena, P. I., Alexeeva, Y. V., Flavier, S., Wright, J. P., Zhukova, N. V., Gorshkova, N. M., Mikhailov, V. V., Nicolau, D. N., and Christen, R. (2004). *Formosa algae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **54**, 705-711.

Ellerbroek, L. (1997). Airborne microflora in poultry slaughtering establishments. *Food Microbiology* **14**, 527-531.

Eneroth, E., Remberger, M., Vahlne, A., and Ringden, O. (2005). Increased serum concentrations of interleukin-2 receptor in the first trimester in women who later developed severe preeclampsia. *Acta Obstetricia of Gynecologica Scandinavica* **77**, 591-593.

Engelbrecht, K., Jooste, P. J., and Prior, B. A. (1996). Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. *South African Journal of Food Science and Nutrition* **8**, 66-71.

Euzéby, J. P. (2007). IJSEM Validation List No. 116. List of new names and new combinations previously effectively, but not validly, published. *International Journal of Systematic and Evolutionary Microbiology* 57, 1371-1373.

Euzéby, J. P. (2008). *List of Prokaryotic Names with Standing in Nomenclature*. http://www.bacterio.cict.fr/classiffamilies.html [accessed on 04/11/2008].

Fankhauser, D. B. (2001). Triple sugar iron agar and its use. http://biology. clc.uc.edu/ fankhauser/Labs/ Microbiology/ Triple_Sugar_Iron/ TSI_Use. htm. Difco Manual, 9th edn. University of Cincinnati Clermont College, Batavia, pp, 166-168.

Fairbain, D., and Law, B. A. (1986). Proteinases of psychrotrophic bacteria, their origin, production, properties, effects and control. *Journal of Dairy Research* **53**, 139-177.

Fent, K. (1996). Ecotoxicology of organotin compounds. *Critical Reviews in Toxicology* **26**, 1-117.

Floodgate, G. D., and Hayes, P. R. (1963). The Adansonian taxonomy of some yellow pigmented marine bacteria. *Journal of General Microbiology* **30**, 237-244.

Frank, J. F. (1997). Milk and dairy products. In: *Food Microbiology: Fundamentals and Frontiers.* eds. M. P. Doyle, L. R. Beuchat, T. J. Montville. American Society for Microbiology, Washington, pp. 101-108.

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

Fredericks, D. N., and Relman, D. A. (1996). Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical and Microbiological Reviews* **9**, 18-33.

Freney, J., Hansen, W., Ploton, C., Menguier, H., Madier, S., Bornstein, N., and Fleurette, J. (1987). Septicemia caused by *Sphingobacterium multivorum*. *Journal of Clinical Microbiology* **25**, 1126-1128.

Gallego, V., García, M. T., and Ventosa, A. (2006). *Chryseobacterium hispanicum* sp. nov., isolated from the drinking water distribution of Sevilla, Spain. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1589-1592.

Garbutt, J. (1997). *Essentials of Food Microbiology*. Arnold, London.

García-López, M. L., Santos, J.-A., and Otero, A. (1998). *The Physiology Attributes of Gram-negative Bacteria Associated with Spoilage of Meat and Meat Products.* Blackie Academic & Professional, London.

Geornaras, I., de Jesus, A. E., van Zyl, E., and von Holy, A. (1996). Bacterial populations associated with poultry processing in a South African abattoir. *Food Microbiology* **13**, 457-465.

Gerhardt, P. (1981). Manual of Methods for General Bacteriology. Washington DC: American Society for Microbiology.

Gherna, R., and Woese, C. R. (1992). A partial phylogenetic analysis of the ''Flavobacter-Bacteroides'' phylum: Basis for taxonomic restructuring. *Systematic and Applied Microbiology* **15**, 513-521.

Gilmour, A., and Rowe, M. T. (1981). Microorganisms associated with milk. In: *Dairy Microbiology*, ed. R. K. Robinson. Applied Science Publishers, London, pp. 35-75.

González, C. J., Santos, J. A., García-López, M. L., and Otero, A. (2000). Psychrobacters and related bacteria in freshwater fish. *Journal of Food Protection* **63**, 315-321.

Gosink, J. J., Woese, C. R., and Staley, J. T. (1998). *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolated polar marine bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group and reclassification of "*Flectobaclillus glomeratus"* as *Polaribacter glomeratus* comb. nov. *International Journal of Systematic Bacteriology* **48**, 223-235.

Gram, L., and Dalgaard, P. (2002). Fish spoilage bacteria – problems and solutions. *Current Opinion in Biotechnology* **13**, 262-266.

Gram, L., and Huss, H. H. (1996). Microbiological spoilage of fish and fish products. *International Journal of Food Microbiology* **33**, 121-137.

Griffiths, M. W., and Phillips, J. D. (1990). Incidence, source and some properties of psychrotrophic bacteria during growth in milk. I. Use of naturally occurring materials. *Milchwissenschaft* **45**, 621-625.

Hantsis-Zacharov, E., and Halpern, M. (2007). *Chryseobacterium haifense* sp. nov., a psychrotolerant bacterium isolated from raw milk. *International Journal of Systematic and Evolutionary Microbiology* **57**, 2344-2348.

Hantsis-Zacharov, E., Senderovich, Y., and Halpern, M. (2008). *Chryseobacterium bovis* sp. nov., isolated from raw cow's milk. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1024-1028.

Harrigan, W.F., and McCance, M. E. (1976). *Laboratory Methods in Food and Dairy Microbiology*. Academic Press, London.

Harrison, F. C. (1929). The discolouration of halibut. *Contributions to Canadian Biology and Fisheries* **1**, 214-239.

Hayes, P. R. (1977). A taxonomic study of flavobacteria and related Gram-negative yellow pigmented rods. *Journal of Applied Bacteriology* **43**, 345-367.

Hayes, P. R. (1985). *Food Microbiology and Hygiene*. Elsevier, London, pp. 80-139.

Herzog, P., Winkler, I., Wolking, D., Kämpfer, P., and 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. *Journal of Systematic and Evolutionary Microbiology* **58**, 26-33.

Holding, A., and Collee, J. G. (1971). Routine biochemical tests. In: *Methods in Microbiology*. eds. J. R. Norris, and D. W. Ribbons. Academic Press, London, pp. 1-32.

Holmes, B. (1992). The genera *Flavobacterium, Sphingobacterium* and *Weeksella*. In: *The Prokaryotes*. eds. A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer. Springer-Verlag. Berlin, pp. 3620-3630.

Holmes, B. (2006). The genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*. In: *The Prokaryotes* Vol 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria 3rd edn., eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H, Schleifer, and E. Stackebrandt. Springer Verlag, New York, pp. 539-548.

Holmes, B., Snell, J. S., and Lapage, S. P. (1977). Revised description, from clinical strains, of *Flavobacterium odoratum* Stutzer and Kwaschnina, 1929

and designation of the neotype strain. *International Journal of Systematic Bacteriology* **27**, 330-336.

Holmes, B., Snell, J. S., and Lapage, S. P. (1978). Revised description, from clinical strains, of *Flavobacterium breve* (Lustig) Bergey *et al*. 1923 and proposal of the neotype strain. *International Journal of Systematic Bacteriology* **28**, 201-208.

Holmes, B., Hollis, D. G., Steigerwalt, A. G., Pickett, M., and Brenner, D. J. (1983). *Flavobacterium thalpophilum*, a new species recovered from human clinical material. *International Journal of Systematic Bacteriology* **33**, 677-682.

Holmes, B., Owen, R. J., and McMeekin, T. A. (1984a). Genus *Flavobacterium* Bergey, Harrison, Breed, Hammer and Huntoon 1923. In: *Bergey's Manual of Systematic Bacteriology*, Volume 1, eds. N.R. Krieg, and J.G. Holt. Williams and Wilkins, Co., Baltimore, pp. 353-361.

Holmes, B., Owen, R. J., Steigerwalt, A. G., and Brenner, D. J. (1984b). *Flavobacterium gleum*, a new species found in human clinical specimens. *International Journal of Systematic Bacteriology*. **34**, 21–25.

Holmes, B., Steigerwalt, A. G., Weaver, R. E., and Brenner, D. J. (1986a). *Weeksella virosa* gen. nov., sp. nov [formerly IIf], found in human clinical specimens. *Systematic and Applied Microbiology* **8**, 185-190.

Holmes, B., Steigerwalt, A. G., Weaver, R. E., and Brenner, D. J. (1986b). *Weeksella zoohelcum* sp. nov. (formerly Group IIj), from human clinical specimens. *Systematic and Applied Microbiology* **8**, 191-196.

Holt, S. C., and Kinder, S. A. (1989). Genus *Capnocytophaga* Leadbetter, Holt and Socransky, 1982. In: *Bergey's Manual of Systematic Bacteriology*. eds. J. T. Staley, M. P. Bryant, N. Pfenning, and J. G, Holt. Williams and Wilkins, Baltimore, pp. 2050-2058.

Hugo, C. J. (1997). *A Taxonomic Study of the Genus Chryseobacterium from Food and Environmental Sources*. Ph. D. Thesis. Department of Microbial, Biochemical and Food Biotechnology. University of the Free State. Bloemfontein.

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

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

Hugo, C. J., and Jooste, P. J. (in press). Culture media for food associated genera in the family *Flavobacteriaceae*. In: *Handbook of Culture Media for Food Microbiology*.

Hugo, C. J., Jooste, P. J., Segers, P., Vancanneyt, M., and Kesters, K. (1999). A polyphasic taxonomic study of *Chryseobacterium* strains isolated from dairy sources. *Systematic and Applied Microbiology* **22**, 586-595.

Hugo, C. J., Segers, P., Hoste, B., Vancanneyt, M., and Kersters, K. (2003). *Chryseobacterium joostei* sp. nov., isolated from the dairy environment. *International Journal of Systematic and Evolutionary Microbiology* **53,** 771-777.

Hugo, C. J., Bruun, B., and Jooste, P.J. (2004a). The Genera *Bergeyella* and *Weeksella*. In: *The Prokaryotes, an Evolving Electronic Resource for the Microbiological Community*. Vol 3. eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. http://141.150.157.117:8080/prokPUB/index.htm.

Hugo, C. J., Bruun, B., and Jooste, P.J. (2004b). The Genera *Empedobacter* and *Myroides*. In: *The Prokaryotes, an Evolving Electronic Resource for the Microbiological Community*. Volume 3. eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. http://141.150.157.117:8080/prokPUB/index.htm.

Hugo, C. J., Bruun, B., and Jooste, P. J. (2006a). Chapter 6.5. The genera *Bergeyella* and *Weeksella*. In: *The Prokaryotes* Vol. 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria, 3rd edn., eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. Springer Verlag, New York, pp. 532-538.

Hugo, C. J., Bruun, B., and Jooste, P. J. (2006b). Chapter 6.11. The genera *Empedobacter* and *Myroides*. In: *The Prokaryotes* Vol. 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria, 3rd edn., eds. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt. Springer Verlag, New York, pp. 630-637.

Huis in't Veld, J. H. J. (1996). Microbial and biochemical spoilage of foods: an overview. *International Journal of Food Microbiology* **33**, 1- 18.

Ivanova, E. P., Nedashkovskaya, O. I., Chun, J., Lysenko, A. M., Frolova, G. M., Svtashev, V. I., Vysotskii, M. V., Mikhailov, V. V., Huq, A., and Colwell, R. R. (2001). *Arenibacter* gen. nov., new genus of the family *Flavobacteriaceae* and description of a new species, *Arenibacter latericius* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1987-1995.

Ivanova, E. P., Alexeeva, Y. V., Flavier, S., Wright, J. P., Zhukova, N. V., Gorshkova, N. M., Mikhailov, V. V., Nicolau, D. V., and Christen, R. (2004). *Formosa algae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **54**, 705-711.

Jae, H. S., Jung-Hyun, L., Hana, Y., Jongsik, C., Kyung, S. B., Tae-Young, A., and Sang-Jin, K. (2004). *Kordia algicida* gen. nov., sp. nov., an *algicidal* bacterium isolated from red tide. *International Journal of Systematic and Evolutionary Microbiology* **54**, 675-680.

Jay, J. M. (1996). *Modern Food Microbiology*, 5th edn. Thomson Publishing, New York.

Jay, J. M., Loessner, M. J., and Golden, D. A. (2003). *Modern Food Microbiology,* 7th edn. Thomson Publishing, New York.

Jenness, R. (1988). Composition of milk. In: *Fundamentals of Dairy Chemistry,* ed. N. P. Wong. Van Nostrand Reinhold, New York, pp. 1-38.

Jeyasekaran, G., Ganesan, P., Anandaraj, R., Jeya Shakila, R., and Sukumar, S. (2006). Quantitative and qualitative studies on the bacteriological quality of Indian white shrimp (*Penaeus indicus*) stored in dry ice. *Food Microbiology* **23**, 526-533.

Johansen, J. E., Nielsen, P., and Sjøholm, C. (1999). Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *International Journal of Systematic Bacteriology* **49**, 1231-1240.

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

Jooste, P. J. (1974). The microbial composition of South African commercial butter before and after keeping quality storage. *South African Journal of Dairy Technology* **6**, 53-60.

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

Jooste, P. J., and Britz, T. J. (1986). The significance of flavobacteria as proteolytic psychrotrophs in milk. *Milchwissenschaft* **41**, 618-621.

Jooste, P. J., and Hugo, C. J. (1999). The taxonomy, ecology and cultivation of bacterial genera belonging to the family *Flavobacteriaceae*. *International Journal of Food Microbiology* **53**, 81-94.

Jooste, P. J., Britz, T. J., and De Haast, J. (1985). A numerical taxonomic study of *Flavobacterium-Cytophaga* strains from dairy sources. *Journal of Applied Bacteriology* **59**, 311-323.

Jooste, P. J., Britz, T. J., and Lategan, P. M. (1986). The prevalence and significance of *Flavobacterium* strains in commercial salted butter. *Journal of Applied Bacteriology* **59**, 311-323.

Jung, S.-Y., Kang, S.-J., Lee, M.-H., Lee, S.-Y., Oh, T.-K., and 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. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1845-1849.

Kämpfer, P., Avesani, V., Janssens, M., Charlier, J., De Baere, T., and Vaneechoutte, M. (2006). Description of *Wautersiella falsenii* gen. nov., sp. nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2323-2329.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2006a). *Krokinobacter* gen. nov., with three novel species in the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 323-328.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2006b). *Sandarakinotalea sediminis* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 959-963.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2006c). *Sediminicola luteus* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 841-845.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2007a). *Galbibacter mesophilus* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 969-973.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2007b). *Sediminibacter furfurosus* gen. nov., sp. nov. and *Gilvibacter sediminis* gen. nov., sp. nov., novel members of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 265-269.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2007c). *Sediminibacter furfurosus* gen. nov., sp. nov. and *Gilvibacter sediminis* gen. nov., sp. nov., novel members of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 265-269.

Khan, S. T., Nakagawa, Y., and Harayama, S. (2008). *Fulvibacter tottoriensis* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1670-1674.

Kim, M. K., Im, W.-T., Shin, Y. K., Lim, J.-H., Kim, S.-H., Lee, B. G., Park, M.-Y., Lee, K. Y., and Lee, S.-K. (2004). *Kaistella koreensis* gen. nov., sp. nov. a novel member of the *Chryseobacterium-Bergeyella-Riemerella* branch.

International Journal of Systematic and Evolutionary Microbiology **54**, 2319-2324.

Kim, K. K., Kim, M.-K., Lim, J.-H., Park, H. Y., and Lee, S.-T. (2005). Transfer of *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to *Elizabethkingia* gen. nov. as *Elizabethkingia meningoseptica* comb. nov. and *Elizabethkingia miricola* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1287-1293.

Kim, K.-H., Ten, L. N., Liu, Q.-M., Im, W.-K., and Lee, S.-T. (2006). *Sphingobacterium daejeonense* sp. nov., isolated from a compost sample. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2031-2036.

Kim, J. H., Kim, K. Y., Hahm, Y. T., Kim, B. S., Chun, J., and Cha, C. J. (2008a). *Actibacter sediminis* gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae* isolated from tidal flat sediment. *International Journal of Systematic and Evolutionary Microbiology* **58**, 139-143.

Kim, K. K., Lee, K. C., Oh, H.-M., and Lee, J.-S. (2008b). *Chryseobacterium aquaticum* sp. nov., isolated from a water reservoir. *International Journal of Systematic and Evolutionary Microbiology* **58**, 533-537.

Kirchman, D. L. (2002). The ecology of *Cytophaga*-flavobacteria in aquatic environments. *FEMS Microbiology Ecology* **39**, 91-100.

Krechel, A., Faupel, A., Hallmann, J., Ulrich, A., and Berg, G. (2002). Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Melaidogyne incognita* (Kofoid & White) Chitwood. *Canadian Journal of Microbiology* **48**, 772-786.

Kumar, S., Dudley, J., Nei, M., and Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* **9**, 299-306.

Kwon, K. K., Lee, Y. K., and Lee, H. K. (2006a). *Costertonia aggregata* gen. nov., sp. nov., a mesophilic marine bacterium of the family *Flavobacteriaceae*, isolated from a mature biofilm. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1349-1353.

Kwon, K. K., Lee, H. S., Jung, H. B., Kang, J. H., and Kim, S. J. (2006b). *Yeosuana aromativorans* gen. nov., sp. nov., a mesophilic marine bacterium belonging to the family *Flavobacteriaceae*, isolated from estuarine sediment of the South Sea, Korea. *International Journal of Systematic and Evolutionary Microbiology* **56**, 727-732.

Labadie, J. (1999). Consequences of packaging on bacterial growth. Meat as an ecological niche. *Meat Science* **52**, 445-448.

Lau, S. C. K., Tsoi, M. M. Y., Li, X., Plakhotnikova, I., Dobretsov, S., Wong, P. K., Pawlik, J. R., and Qian, P. Y. (2005). *Nonlabens tegetincola* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a microbial mat in a subtropical estuary. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2279-2283.

Lau, S. C. K., Tsoi, M. M. Y., Li, X., Plakhotnikova, I., Dobretsov, S., Wu, M., Wong, P. K., Pawlik, J. R., and Qian, P. Y. (2006). *Stenothermobacter spongiae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a marine sponge in the Bahamas, and emended description of *Nonlabens tegetincola*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 181-185.

Leadbetter, E. R., Holt, S. C., and Socransky, S. S. (1979). *Capnocytophaga*: new genus of Gram-negative gliding bacteria. I. General characteristics, taxonomic considerations and significance. *Archives of Microbiology* **122**, 9–16.

Leclerc, H., and Moreau, A. (2002). Microbiological safety of natural mineral water. *FEMS Microbiology Revie*ws **6**, 207-222.

Lee, S. D. (2007). *Tamlana crocina* gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae*, isolated from beach sediment in Korea. *International Journal of Systematic and Evolutionary Microbiology* **57**, 764-769.

Lee, H. S., Kwon, K. K., Yang, S. H., Bae, S. S., Park, C. H., Kim, S. J., and Lee, J. H. (2008). Description of *Croceitalea* gen. nov. in the family *Flavobacteriaceae* with two species, *Croceitalea eckloniae* sp. nov. and *Croceitalea dokdonensis* sp. nov., isolated from the rhizosphere of the marine alga *Ecklonia kurome*. *International Journal of Systematic and Evolutionary Microbiology* **58**, 2505-2510.

Lewin, R. A., and Lounsbery, D. M. (1969). Isolation, cultivation and characterization of flavobacteria. *Journal of General Microbiology* **58**, 145-170.

Liao, C. H. (1989). Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. *Applied and Environmental Microbiology* **7**, 1677-1683.

Liao, C. H., and Fett, W. F. (2001). Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *Journal of Food Protection* **64**, 1110-1115.

Liao, C. H., Sullivan, J., Grady, J., and Wong, J. L. C. (1997). Biochemical characterization of pectate lyases produced by fluorescent *Pseudomonas* associated with spoilage of fresh fruits and vegetables. *Applied and Environmental Microbiology* **83**, 10-16.

Lim, J. M., Jeon, C. O., Lee, S. S., Park, D. J., Xu, L. H., Jiang, C. L., and Kim, C. J. (2008). Reclassification of *Salegentibacter catena* Ying *et al*. 2007

as *Salinimicrobium catena* gen. nov., comb. nov. and description of *Salinimicrobium xinjiangense* sp. nov., a halophilic bacterium isolated from Xinjiang province in China. *International Journal of Systematic and Evolutionary Microbiology* **58**, 438-442.

Liu, Z. P., Wang, B. J., Dai, X., Liu, X. Y., and Liu, S. J. (2006). *Zhouia amylolytica* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from sediment of the South China Sea. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2825-2829.

Liu, R., Liu, H., Zhang, C. X., Yang, S. Y., Liu, X. H., Zhang, K. Y., and Lai, R. (2008). *Sphingobacterium siyangense* sp. nov., isolated from farm soil. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1458-1462.

Lopez, M. J., Nichols, N. N., Dien, B. S., Moreno, J., and Bothast, R. J. (2004). Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. *Applied Microbiology and Biotechnology* **64**, 125-131.

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

Macrae, A. (2000). The use of 16S rDNA methods in soil microbial ecology. *Brazilian Journal of Microbiology* **31**, 77-82.

Manani, T. A., Collison, E. K., and Mpuchane, S. (2006). Microflora of minimally processed frozen vegetables sold in Gaborone, Botswana. *Journal of Food Protection* **69**, 2581-2586.

Mancusa Nichols, C. M., Bowman, J. P., and Gaenzemec, J. (2005). *Olleya marilimosa* gen. gen., sp. nov., an exopolysaccharide-producing marine bacterium from the family *Flavobacteriaceae*, isolated from the Southern

Ocean. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1557-1561.

Manz, W., Wendt-Potthof, K., Neu, T. R., Szewzyk, U., and 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 Ecology* **37**, 225-237.

Matsuyama, H., Katoh, H., Ohkushi, T., Satoh, A., Kawahara, K., and Yumoto, I. (2008). *Sphingobacterium kitahiroshimense* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1576-1579.

Mattila, P., Salo-Vaananen, P., Kanko, K., Aro, H., and Jalava, H. (2002). Basic composition and amino acid content of mushrooms cultivated in Finland. *Journal of Agricultural and Food Chemistry* **50**, 6419-6422.

McCammon, S. A., and Bowman, J. P. (2000). Taxonomy of Antarctic *Flavobacterium* species: description of *Flavobacterium gillisiae* sp. nov., *Flavobacterium tegetincola* sp. nov. and *Flavobacterium xanthum* sp. nov., nom. rev., and reclassification of [*Flavobacterium*] *salegens* as *Salegentibacter salegens* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **50**, 1055–1063.

McMeekin, T. A., Patterson, J. T., and Murray, J. G. (1971). An initial approach to the taxonomy of some Gram-negative yellow pigmented rods. *Journal of Applied Bacteriology* **34**, 699-716.

McMeekin, T. A., Steward, D. B., and Murray, J. G. (1972). The Adansonian taxonomy and the deoxyribonucleic acid base composition of some Gram-negative, yellow pigmented rods. *Journal of Applied Bacteriology* **35**, 129-137.

Meer, R. R., Baker, J., Bodyfelt, F. W., and Griffiths, M. W. (1991). Psychrotrophic *Bacillus* spp. in fluid milk products: a review. *International Journal of Food Protection* **54**, 969-979.

Mielmann, A. (2006). *Food Spoilage Characteristics of Chryseobacterium Species.* M.Sc. Thesis. University of the Free State, Bloemfontein, South Africa.

Mignard, S., and Flandrois, J. P. (2006). 16S rRNA sequencing in routine bacterial identification: 30-month experiment. *Journal of Microbiological Methods* **67**, 574-581.

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

Mudarris, M., and Austin, B. (1989). Systematic disease in turbot (*Scophthalmus maximus*) caused by a previously unrecognised *Cytophaga*-like bacterium. *Disease of Aquatic Organisms* **6**, 161-166.

Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B., and Bernardet, J.-F. (1994). *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *International Journal of Systematic Bacteriology* **44**, 447-453.

Nakagawa, Y., and Yamasato, K. (1993). Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis. *Journal of General Microbiology* **139**, 1155-1161.

Nakagawa, Y., Sakane, T., Suzuki, M., and Hatano, K. (2002). Phylogenetic structure of the genera *Flexibacter*, *Flexithrix*, and *Microscilla* deduced from 16S rRNA sequence analysis. *Journal of General and Applied Microbiology* **48**, 155-165.

Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Lysenko, M., Rohde, M., Zhukova, N., Falsen, E., Frolova, G. M., Mikhailov, V. V., and Bae, K. S. (2003a). *Mesonia algae* gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae* isolated from the green alga *Acrosiphonia sonderi* (Kutz) Kornm. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1967-1971.

Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Rhee, A. M., Lysenko, E., Falsen, E., Frolova, G. M., Mikhailov, V. V., and Bae, K. S. (2003b). *Ulvibacter litoralis* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from the green algae *Ulva fenestrans*. *International Journal of Systematic and Evolutionary Microbiology* **54**, 119-123.

Nedashkovskaya, O. I., Suzuki, M., Vysotskii, M. V., and Mikhailov, V. V. (2003c). *Arenibacter troitsensis* sp. nov., isolated from marine bottom sediment. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1287-1290.

Nedashkovskaya, O. I., Suzuki, M., Vysotskii, V., and Mikhailov, V. V. (2003d). *Vitellibacter vladivostokensis* gen. nov., sp. nov., a new member of the phylum *Cytophaga-Flavobacterium-Bacteroides*. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1281-1286.

Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Rhee, M., Lysenko, A. M., Rohde, M., Zhukova, N. V., Frolova, G. M., Mikhailov, V. V., and Bae, K. S. (2004). *Maribacter* gen. nov., a new member of the family *Flavobacteriaceae*, isolated from marine habitats, containing the species *Maribacter sedimenticola* sp. nov., *Maribacter aquivivus* sp. nov., *Maribacter orientalis* sp. nov. and *Maribacter ulvicola* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1017-1023.

Nedashkovskaya, O. I., Kim, S. B., Lysenko, A. M., Frolova, G. M., Mikhailov, V. V., Lee, K. H., and Bae, K. S. (2005a). Description of

Aquimarina muelleri gen. nov., sp. nov., and proposal of the reclassification of [*Cytophaga] latercula* Lewin 1969 as *Stanierella latercula* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **55**, 225-229.

Nedashkovskaya, O. I., Kim, S. B., Lysenko, A. M., Frolova, G. M., Mikhailov, V. V., and Bae, K. S. (2005b). *Bizionia paragorgiae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from the soft coral *Paragorgia arborea*. *International Journal of Systematic and Evolutionary Microbiology* **55**, 375-378.

Nedashkovskaya, O. I., Kim, S. B., Lee, K. H., Bae, K. S., Frolova, G. M., Mikhailov, V. V., and Kim, I. S. (2005c). *Pibocella ponti* gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae* isolated from the green alga *Acrosiphonia sonderi*. *International Journal of Systematic and Evolutionary Microbiology* **55**, 177-181.

Nedashkovskaya, O. I., Kim, S. B., Kwak, J., Mikhailov, V. V. and Bae, K. S. (2006). *Mariniflexile gromovii* gen. nov., sp. nov., a gliding bacterium isolated from the sea urchin *Strongylocentrotus intermedius*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1635-1638.

Nedashkovskaya, O. I., Vancanneyt, M., Kim, S.-B., Hoste, B., and Bae, K.-S. (2007). *Algibacter mikhailovii* sp. nov., a novel marine bacterium of the family *Flavobacteriaceae*, and emended description of the genus *Algibacter*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 2147-2150.

Nedashkovskaya, O. I., Suzuki, M., Kim, S. B., and Mikhailov, V. V. (2008). *Kriegella aquimaris* gen. nov., sp. nov., isolated from marine environments. *International Journal of Systematic and Evolutionary Microbiology* **58**, 2624-2628.

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

Olofsson, T. C., Ahrné, S., and Molin, G. (2007). Composition of the bacterial population of refrigerated beef, identified with direct 16S rRNA gene analysis and pure culture techniques. *International Journal of Food Microbiology* **118**, 233-240.

Owen, R. J., and Holmes, B. (1980). Differentiation between strains of *Flavobacterium breve* and allied bacteria by comparisons of deoxyribonucleic acids. *Current Microbiology* **4**, 7-11.

Park, M. S., Jung, S. R., Lee, M.-S., Kim, K. O., Do, J. O., Lee, K. H., Kim, S. B., and Bae, K. S. (2006). *Chryseobacterium soldanellicola* sp. nov. and *Chryseobacterium taeanense* sp. nov. isolated from roots of sand dune plants. *International Journal of Systematic and Evolutionary Microbiology* **56**, 433-438.

Pavlov, D., de Wet, C. M. E., Grabow, W. O. K., and Ehlers, M. M. (2004). Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *International Journal of Systematic and Evolutionary Microbiology* **92**, 275-287.

Pinhassi, J., Bowman, J. P., Nedashkovskaya, O. I., Lekunberri, I., Gomez-Consarnau, L., and Pedrós-Alio, C. (2006). *Leeuwenhoekiella blandensis* sp. nov., a genome-sequenced marine member of the family *Flavobacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1489-1493.

Preston-Mafham, J., Boddy, L., and Randerson, P. F. (2002). Analysis of microbial community functional diversity using sole-carbon-source utilization profiles - a critique. *FEMS Microbiology Ecology* **42**, 1-14.

Qin, Q. L., Zhao, D. L., Wang, J., Chen, X. L., Dang, H. Y., Li, T. G., Zhang, Y. Z., and Gao, P. J. (2007). *Wangia profunda* gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae* isolated from southern Okinawa Trough deep-sea sediment. *FEMS Microbiology Letters* **271**, 53-58.

Quan, Z. X., Xiao, Y. P., Roh, S. W., Nam, Y. D., Chang, H. W., Shin, K. S., Rhee, S. K., Park, Y. H., and Bae, J. W. (2008). *Joostella marina* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from the East Sea. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1388-1392.

Ravenschlag, H., and Dworkin, M. (2001). Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). *Applied and Environmental Microbiology* **67**, 387-395.

Reichenbach, H. (1989). Order I. *Cytophagales* Leadbetter 1974, 99^{AL} In: *Bergey's Manual of Determinative Bacteriology*, Volume 3, ed. J. T. Staley, M. P. Bryant, N. Pfenning, and J. G. Holt. Williams & Wilkins, Co., Baltimore, pp. 2010-2082.

Reichenbach, H. (1992). *Flavobacteriaceae* fam. nov.: Validation of the publication of new names and new combinations previously effectively published outside the IJSB, List no. 41. *International Journal of Systematic Bacteriology* **42**, 327-329.

Reina, J., Borrell, N., and Figuerola, J. (1992). *Sphingobacterium multivorum* isolated from a patient with cystic fibrosis. *European Journal of Clinical Microbiology and Infectious Disease* **11**, 81-82.

Romanenko, L. A., Uchino, M., Frolova, G. M., and Mikhailov, V. V. (2007). *Marixanthomonas ophiurae* gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae* isolated from a deep-sea brittle star. *International Journal of Systematic and Evolutionary Microbiology* **57**, 457-462.

Roussis, I. G., Matselis, E., Papamichael, E. M., Sakarellos-Daitsotis, M., and Trianttafyllidou, M. (1999). Extracellular proteinases and lipases activity of *Flavobacterium* MTR3. *Milchwissenschaft* **54**, 77-81.

Sakai, T., Kimura, H., and Kato, I. (2002). A marine strain of *Flavobacteriaceae* utilizes brown seaweed fucoidan. *Marine Biotechnology* **4**, 406-425.

Samina, M., Weselowski, B., and Lazarovits, G. (2007). *Sphingobacterium canadense* sp. nov., an isolate from corn roots. *Systematic and Applied Microbiology* **30,** 519-524.

Sastry, S. K., Li, S. F., Patel, P., Konanayakam, M., Bafna, P., Doores, S., and Beelman, R. B. (2006). A bioindicator for verification of thermal processes for particulate foods. *Journal of Food Science* **53**, 1528-1536.

Schröder, M. J. A. (1984). Origins and levels of post pasteurization contamination of milk in the dairy and their effects on keeping quality. *Journal of Dairy Research* **51**, 59-67.

Segers, P., Mannheim, W., Vancanneyt, M., De Brandt, K., Hinz, K.-H., Kersters, K., and Vandamme, P. (1993). *Riemerella anatipestifer* gen. nov., comb. nov., the causative agent of septicemia anserum exsudative, and its phylogenetic affiliation within the *Flavobacterium-Cytophaga* rRNA homology group. *International Journal of Systematic Bacteriology* **43**, 768-776.

Shen, F. T., Kämpfer, P., Young, C. C., Lai, W. A., and Arun, A. B. (2005). *Chryseobacterium taichungense* sp. nov., isolated from contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1310-1304.

Shimomura, K., Kaji, S., and Hiraishi, A. (2005). *Chryseobacterium shigense* sp. nov., a yellow-pigmented, aerobic bacterium isolates from a

lactic acid beverage. *International Journal of Systematic and Evolutionary Microbiology* **55**, 1903-1906.

Shivaji, S., Ray, M. K., Rao, N. S., Saisree, L., Jagannadham, M. V., Kumar, G. S., Reddy, G. S., and Bhargava, P. M. (1992). *Sphingobacterium antarcticus* sp. nov., a psychrotrophic bacterium from the soil of Schirmacher Oasis, Antarctica. *International Journal of Systematic Bacteriology* **42,** 102-106.

Smith, R., Nickelson, R., Martin, R., and Finne, G. (1984). Bacteriology of indole production in shrimp homogenates held at different temperatures. *Journal of Food Protection* **47**, 861-864.

Sohn, J. H., Lee, J.-H., Yi, H., Chun, J., Bae, K. S., Ahn, T.-K., and Kim, S.-J. (2004). *Kordia algicida* gen. nov., sp. nov., an algacidal bacterium isolated from red tide. *International Journal of Systematic and Evolutionary Microbiology* **54**, 675-680.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, Maiden, M. C., Nesme, X., Rossello-Mora, R., Swings, J., Truper, H. G., Vauterin, L., Ward, A. C., and Whitman, W. B. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1043-1047.

Stanley, C. K. L., Tsoi, M. M. Y., Li, X., Plakhotnikova, I., Dobretsov, S., Wong, P.-K., and Qian, P.-Y. (2005). *Gramella portivictoriae* sp. nov., a novel member of the family *Flavobacteriaceae* isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2497-2500.

Steyn, P. L., Segers, P., Vancanneyt, P., Sandra, K., and Joubert, J. J. (1998). Classfication of heparinolytic bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus* comb. nov., *Pedobacter*

piscium comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. Proposal of the family *Sphingobacteriaceae* fam. nov*. International Journal of Systematic Bacteriology* **48**, 165-177.

Suzuki, M., Nakagawa, Y., Harayama, S., and 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. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1639-1652.

Tatum, H. W., Ewing, W. H., and Weaver, R. E. (1974). Miscellaneous Gram-negative bacteria. In: *Manual of Clinical Microbiology*, 2nd edn., ed. E. H. Lennette, E. H. Spaulding, and J. P. Traunt. American Society for Microbiology, Washington DC, pp. 270-294.

Tournas, V. H. (2005). Spoilage of vegetables crops by bacteria and fungi and related health hazards. *Critical Reviews in Microbiology* **31**, 33-44.

Ultee, A., Souvatzi, N., Maniadi, K., and König, H. (2004). Identification of the culturable and nonculturable bacterial population in ground water of a municipal water supply in Germany. *Journal of Applied Microbiology* **96**, 560-568.

Urbach, G. (1997). The flavour of milk and dairy products: ll. Cheese: contribution of volatile compounds. *International Journal of Dairy Technology* **50**, 79-89.

Ursing, J., and Bruun, B. (1991). Genetic heterogeneity of *Flavobacterium* group IIb and *Flavobacterium breve* demonstrated by DNA-DNA hybridization. *Acta Pathalogica, Microbiologica et Immunologica. Scandinavica. Section B* **99**, 780-786.

Vancanneyt, M., Segers, P., Torck, U., Hoste, B., Bernadet, J.-F., Vandamme, P., and Kersters, K. (1996). Reclassification of *Flavobacterium odoratum* (Stutzer 1929) strains to a new genus, *Myroides*, as *Myroides odoratum* comb. nov. and *Myroides odoratimimus* sp. nov. *International Journal of Systematic Bacteriology* **46**, 926-932.

Vandamme, P., Bernardet, J.-F., Segers, P., Kersters, K., and Holmes, B. (1994a). New perspectives in the classification of the flavobacteria: description of *Chryseobacterium* gen. nov., *Bergeyella*, gen. nov., and *Empedobacter* nom. rev. *International Journal of Systematic Bacteriology* **44**, 827-831.

Vandamme, P., Segers, P., Vancanneyt, M., Van Hove, K., Mutters, R., Hommez, J., Dewhirst, F., Paster, B., Kesters, K., and Falsen, E. (1994b). *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from the avian respiratory tract. *International Journal of Systematic Bacteriology* **44**, 24-37.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., and Swings, J. (1996a). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews* **60**, 407-438.

Vandamme, P., Vancanneyt, M., van Belkum, A., Segers, P., Quint, W. G. V., Kersters, K., Paster, B., and Dewhirst, F. E. (1996b). Polyphasic analysis of strains of the genus *Capnocytophaga* and Center for Disease Control Group DF-3. *International Journal of Systematic Bacteriology* **46**, 782-791.

Vandamme, P., Vancanneyt, M., Segers, P., Ryll, M., Kohler, B., Ludwig, W., and Hinz, K. H. (1999). *Coenonia anatina* gen. nov., sp. nov., a novel bacterium associated with respiratory disease in ducks and geese. *International Journal of Systematic Bacteriology* **49**, 867-874.

Van Trappen, S., Vandecandelaere, I., Mergaert, J., and Swings, J. (2004). *Gillisia limnaea* gen. nov., sp. nov., a new member of the family

Flavobacteriaceae isolated from a microbial mat in Lake Fryxell, Antartica. *International Journal of Systematic and Evolutionary Microbiology* **54**, 445-448.

Van Wyk, E. R. (2008). *Virulence Factors and Other Clinical Relevant Characteristics of Chryseobacterium Species*. M. Sc. thesis. Department of Microbial, Biochemical and Food Biotechnology. University of the Free State. Bloemfontein.

Venter, H., Osthoff, G., and Litthauer, D. (1999). Purification and characterization of a metalloprotease from *Chryseobacterium indologenes* Ix9a and determination of the amino acid specificity with electrospray mass spectrometry. *Protein Expression and Purification* **15**, 282–295.

Von Graevenitz, A. (1995). *Acinetobacter, Alcaligenes, Moraxella* and other nonfermentative Gram-negative bacteria. In: *Manual of Clinical Microbiology,* 6th edn, eds. P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken. ASM Press, Washington DC, pp. 522-532.

Volossiouk, T., Robb, E. J., and Nazar, R. N. (1995). Direct DNA extraction for PCR-mediated assays of soil organisms. *Applied and Environmental Microbiology* **61**, 3972-3976.

Wakabayashi, H., Hikida, M., and Masumura, K. (1986). *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *International Journal of Systematic Bacteriology* **39**, 213-216.

Walker, S. J. (2007). Major spoilage microorganisms in milk and dairy products. *International Journal of Dairy Technology* **41**, 91-92.

Walker, S. J., and Stringer, M. F. (1990). Microbiology of chilled foods. In: *Chilled Foods: the State of the Art*, ed. T. M. Gormley. Elsevier Science, United Kingdom, pp. 269-304.

Wei, W., Zhou, Y., Wang, X., Huang, X., and Lai, R. (2008). *Sphingobacterium anhuiense* sp. nov., isolated from forest soil. *International Journal of Systematic and Evolutionary Microbiology* **58**, 2098-2101.

Welthagen, J. J., and Jooste, P. J. (1992). Isolasie en karakterisering van gepigmenteerde psigrotrofe backteriee uit verkoelde roumelk. *South African Journal of Dairy Science* **24**, 47-52.

Weon, H.-Y., Kim, B.-K., Yoo, S.-H., Kwon, S.-W., Cho, Y.-H., Go, S.-J., and Stackebrandt, E. (2006*).* *Chryseobacterium wanjuense* sp. nov., isolated from greenhouse soil in Korea. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1501-1504.

Weon, H.-Y., Song, M.-Y., Son, J. A., Kim, Y.-K., Kwon, S.-W., Go, S.-Y., and Stackebrandt, E. (2007). *Flavobacterium terrae* sp. nov. and *Flavobacterium cucumis* sp. nov., isolates from greenhouse soil. *International Journal of Systematic and Evolutionary Microbiology* **57**, 1594-1598.

Whiteley, A. S., and Bailey, M. J. (2000). Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Applied and Environmental Microbiology* **66**, 2400-2407.

Wolochow, H., Thorton, H. R., and Hood, E. G. (1942). Studies on surface taint butter. VI. Other bacterial species as causal agents. *Flavobacterium maloloris* (n. sp). *Scientific Agriculture* **22**, 637-644.

Yabuuchi, E., Kaneko, T., Yano, I., Moss, C. W., and Miyoshi, N. (1983). *Sphingobacterium* gen. nov., *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb. nov., *Sphingobacterium mizutae* sp. nov.: glucose-nonfermenting gram-negative rods in CDC groups llk-2 and llb. *International Journal of Systematic Bacteriology* **33**, 580-598.

Yabuuchi, E., Hashimoto, Y., Ezaki, T., Ido, Y., and Takeuchi, N. (1990). Genotypic and phenotypic differentiation of *Flavobacterium indologenes* Yabuuchi *et al*., 1983 from *Flavobacterium gleum* Holmes *et al*., 1984. *Microbiology and Immunology* **34**, 73-76.

Yamaguchi, S., and Yokoe, M. (2000). A novel protein deamidating enzyme from *Chryseobacterium proteolyticum* sp. nov., a newly isolated bacterium from soil. *Applied and Environmental Microbiology* **66**, 3337-3343.

Yang, S. J., Choo, Y. J., and Cho, J. C. (2007). *Lutimonas vermicola* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from the marine polychaete *Periserrula leucophryna*. *International Journal of Systematic and Evolutionary Microbiology* **57**, 1679-1684.

Yi, H., Yoon, H. I., and Chun, J. (2005). *Senjonia antarctica* gen. nov., sp. nov., *Senjonia jeonii* sp. nov., isolated from the Antarctic. *International Journal of Systematic and Evolutionary Microbiology* **55**, 423-426.

Yoo, S.-H., Weon, H.-Y., Jang, H.-B., Kim, B.-Y., Kwon, S.-W, Go, S.-J., and Stackebrandt, E. (2007). *Sphingobacterium composti* sp. nov., isolated from cotton-waste compost. *International Journal of Systematic Bacteriology* **57**, 1590-1593.

Yoon, J.-H., Kang, S.-J., Lee, C.-H., and Oh, T.-K. (2005). *Dokdonia donghaensis* gen. nov., sp. nov., isolated from sea water. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2323-2328.

Yoon, J.-H., Kang, S.-J., Jung, S.-Y., Oh, H. W., and Oh, T.-K. (2006a). *Gaetbulimicrobium brevivitae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a tidal flat of the yellow sea in Korea. *International Journal of Systematic and Evolutionary Microbiology* **56**, 115-119.

Yoon, J.-H., Kang, S.-J., Lee, C.-H., and Oh, T.-K. (2006b). *Donghaeana dokdonensis* gen. nov., sp. nov., isolated from sea water. *International Journal of Systematic and Evolutionary Microbiology* **56**, 187-191.

Yoon, J. H., Kang, S. J., Jung, Y. T., and Oh, T. K. (2008). *Aestuariicola saemankumensis* gen. nov., sp. nov., a member of the family *Flavobacteriaceae*, isolated from tidal flat sediment. *International Journal of Systematic and Evolutionary Microbiology* **58**, 2126-2131.

Young, C. C., Kämpfer, P., Shen, F. T., Lai, W. A., and Arun, A. B. (2005). *Chryseobacterium formosense* sp. nov., isolated from the rhizosphere of *Lactuca sativa* (garden lettuce). *International Journal of Systematic and Evolutionary Microbiology* **55**, 409-426.

CHAPTER 7

SUMMARY

Frequent reports of yellow-pigmented Gram-negative rods being encountered during routine bacteriological analyses of milk and milk products, meat, canned products and surface waters, accentuate the universal distribution of *Flavobacteriaceae*. Many organisms previously regarded as *Flavobacterium* have been found to belong to several new genera in the family *Flavobacteriaceae*. The introduction of a chemotaxonomic approach and molecular techniques such as rRNA sequencing, has made it possible to refine the differentiation between closely related genera.

Although *Flavobacteriaceae* are widely distributed in soil and plants, their presence and roles in these environments has received less attention in South Africa. The first purpose of this study was to investigate the presence of *Flavobacteriaceae* from eight different vegetables and the surrounding soil in which the vegetables were planted; yellow pigmented Gram-negative bacteria were isolated from the samples and examined for the presence of *Flavobacteriaceae*. The second part of this study was to taxonomically re-define flavobacterial dairy isolates from previous studies using newer phenotypic procedures, including the BIOLOG system and also genotyping techniques such as 16S rRNA sequencing.

Phenotypic tests were used for the preliminary grouping of isolates. The BIOLOG Microplate system showed that most “flavobacterial” isolates from vegetables were highly related to the genus *Sphingobacterium*, whereas isolates from soil were related to *Chryseobacterium gleum/indologenes*. Most of the dairy isolates identified with this method were closely related to the genera *Chryseobacterium* and *Empedobacter*. The 2001 database of the BIOLOG

system currently in use, is however, incapable of identifying all *Flavobacteriaceae* genera and species since no new additions have been made and it is also not possible to manually do so.

Isolates were subsequently subjected to the 16S rRNA sequencing technique which was able to verify and differentiate organisms identified by the BIOLOG Microplate system. Because of poor quality DNA, the two soil isolates could not be identified with this method. Fourteen of the 18 dairy isolates that underwent sequencing were highly related to the genus *Chryseobacterium* while four isolates were identified as *E. brevis*.

The possible spoilage characteristics of the *Flavobacteriaceae* isolates in this study were also evaluated using the BIOLOG system. It became clear that they could utilize certain carbohydrates, carboxylic acids, amino acids and polymers that could contribute to spoilage defects of food by creating off-odours, off-tastes, sliminess and bitterness of specific foods.

Key words: *Flavobacteriaceae*, spoilage, taxonomy, dairy, vegetables, soil.

HOOFSTUK 7

OPSOMMING

Gereelde bevindings van geel gepigmenteerde Gram-negatiewe stawe gedurende roetiene bakteriologiese analises van melk en melkprodukte, vleis, geblikte produkte en oppervlakwater, beklemtoon die universele verspreiding van *Flavobacteriaceae*. Baie organismes voorheen bekend as *Flavobacterium* behoort nou aan nuwe genera in die *Flavobacteriaceae* familie. Die bekendstelling van ‘n nuwer chemotaksonomiese benadering en molekulêre tegnieke soos rRNA sekwensering, het dit moontlik gemaak om die onderskeid tussen naasbestaande genera te verfyn.

Alhoewel *Flavobacteriaceae* verspreid voorkom in grond en plante, het hulle teenwoordigheid en betekenis in hierdie omgewings minder aandag geniet in Suid-Afrika. Die eerste doel van hierdie studie was om die teenwoordigheid van *Flavobacteriaceae* lede op agt verskillende groentes en omliggende grond waarin groente geplant was, te bepaal. Geel gepigmenteerde Gram-negatiewe bakterieë is geïsoleer en gekarakteriseer. Die tweede deel van hierdie studie was gemik op suiwelisolate uit vorige studies wat voorlopig geïdentifiseer was as *C. balustinum*-agtig, *Empedobacter*-agtig en *Weeksella*-agtig. ‘n Ongeïdentifiseerde SDS-PAGE groep uit so ‘n vorige studie is ook geteiken. In hierdie deel van die huidige studie is die BIOLOG fenotipiese sisteem en 16S rRNA sekwensering toegepas op die bakteriestamme.

Gewone fenotipiese toetse is vir die voorlopige groepering van isolate gebruik. Die BIOLOG Microplate sisteem is vir meer gevorderde identifikasie gebruik en dit het getoon dat die meeste isolate vanaf die groentesoorte hoogs verwant was aan die genus *Sphingobacterium*, terwyl isolate uit die grond verwant was aan *Chryseobacterium gleum/indologenes*. Die meeste van die

suiwelisolate wat met hierdie metode geïdentifiseer is, was na-verwant aan die *Chryseobacterium* en *Empedobacter* genera Die 2001 databasis van die BIOLOG sisteem tans in gebruik, is egter onvoldoende om nuwer *Flavobacteriaceae* genera en species te identifiseer aangesien die stelsel nie ontwerp is om met nuwer data opgegradeer te word nie.

Isolate is daarna aan 16S rRNA sekwensering onderwerp wat instaat was daartoe om die organisms wat met die BIOLOG sisteem geïdentifiseer is, te verifieer en te differensieer. As gevolg van ‘n swak gehalte DNA van die twee grond isolate, kon dit nie met hierdie metode identifiseer word nie. Veertien van die 18 suiwelisolate wat sekwensering ondergaan het, was hoogs verwant aan die *Chryseobacterium* genus terwyl vier isolate as *E. brevis* identifiseer is.

Derdens is die moontlike bederf eienskappe van die *Flavobacteriaceae* isolate in hierdie studie ge-evalueer met behulp van die BIOLOG sisteem. Dit het duidelik geword dat die isolate spesifieke koolhidrate, karboksielsure, aminosure en polimere kon gebruik wat kon bydra tot gebreke in voedsel deur die onstaan van afgeure, afsmake, slymerigheid en bitterheid van spesifieke voedsel te veroorsaak.

Sleutelwoorde: *Flavobacteriaceae*, bederf, taksonomie, suiwel, groente, grond.