A TAXONOMIC STUDY OF *Chryseobacterium* SPECIES IN MEAT

by

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To my husband, Deon and my children, Deon and Elani

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LIST OF ABBREVIATIONS

AHLs	Acylated Homoserine Lactones
ATCC	American Type Culture Collection
B.	*Bergeyella*
C.	*Chryseobacterium*
CCUG	Culture Collection, University of Götenborg
DNA	Deoxyribonucleic acid
F.	*Flavobacterium*
E.	*Elizabethkingia*
FAME	Fatty acid methyl ester
Fig.	Figure
g	Gram
h	hour(s)
LMG	Laboratory for Microbiology, University of Ghent
M.	*Myroides*
mA	milli Ampere
mg	milligram(s)
min	minute(s)
ml	Millilitre
mm	Millimetre
n	Number
r	Pearson correlation coefficient
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
s	Second
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSO	Specific spoilage organisms
STB	Sample Treatment Buffer
TEMED	N,N,N',N'-tetra-methylethylene diamine
U	International unit
μg	Microgram
μl	Microlitre

μm	Micrometer
V	Volt
v/v	volume per volume
v/w	volume per weight

CHAPTER 1

INTRODUCTION

When God created the earth and everything else in the beginning, there was order and man wished to maintain that order. The aim of bacterial taxonomy is to provide the orderly arrangement or grouping of organisms on the basis of their characteristics (Austin & Priest, 1986). The introduction of molecular microbiology gave a new dimension to information technology and gave scientists a better understanding of bacterial taxonomy. Techniques such as PCR technology, RNA sequencing and oligonucleotide probing introduced a new world of information to enable scientists to classify organisms according to their genetic characteristics (Stackebrandt & Goebel, 1994).

The past decade has been characterised by major changes in bacterial taxonomy and the taxonomy of the flavobacterial group of organisms provides an excellent example. Currently the genus *Flavobacterium* is only one of several Gram-negative genera in the *Flavobacteriaceae* family. Significant genera in terms of food microbiology in this family include *Bergeyella, Chryseobacterium, Empedobacter, Myroides* and *Weeksella* (Hugo & Jooste, 2003). Some erstwhile "*Flavobacterium*" species have been included in the new genus *Chryseobacterium* (Bernardet *et al*., 1996). Several new *Chryseobacterium* species have been described of which *C. joostei* (Hugo *et al*., 2003), *C. defluvii* (Kämpher *et al*., 2003) and *C. miricola* (Li *et al.,* 2003) are the latest validated members. During the past six months, three new species were proposed and two *Chryseobacterium* species were relocated to a new genus namely, *Elizabethkingia* (Kim *et al*., 2005b). Organisms that have not been included in the present study are the newest validated species of the *Chryseobacterium* genus, namely *C. formosense* (Young *et al*., 2005), *C. daecheongense* (Kim *et al*., 2005a) and *C. taichungense* (Shen *et al*., 2005) and also '*C. proteolyticum',* a species that has not been validly published (Bernardet *et al*., 2002).

Unfortunately, molecular techniques have their limitations and certain phenotypic characteristics of bacteria are valuable for identification purposes in applied research such as the microbial ecology of food spoilage, a field of study that still poses many unanswered questions. The high protein content of meat and fish gives these products a predictively perishable character and spoilage takes place even under refrigerated conditions. During slaughtering and processing practices, the so-called "sterile" meat is exposed to several environmental and enteropathogenic organisms that are responsible for deterioration of the meat during refrigerated storage conditions or pose a hazard of food-borne disease (Forsythe, 2000).

Spoilage of raw meat will result in undesirable off-odours and flavours due to metabolic end products, possible slime production and discolouration of specific surfaces of the product (Labadie, 1999). This in turn will have an economic impact on processor and consumer alike. Metabolites produced by flavobacteria include alcohols, sulphur compounds, ketones, aldehydes, esters and amines and the resultant odours can be described as fishy, foul, sulphuric and ammonia like (Nychas & Drosinos, 1999).

The changing taxonomy of the flavobacteria and their proven and potential significance as food spoilage organisms has been a prime motivation for further research. The main purpose of this study, therefore, was to determine the taxonomic heterogeneity of flavobacterial strains occurring in poultry, red meat and fish. This was deemed necessary to, in the end, have a better understanding of their origin, characteristics and potential significance, not only in these food commodities, but in general. As environmental organisms, they may be shown to contribute to spoilage of these foods or they may later prove to be opportunistic pathogens in humans and animals. Better knowledge of these organisms may even demonstrate a beneficial role in some as yet unknown way.

The first objective in this study was to isolate a range of flavobacterial strains from red meat, poultry and fish over a 15-month period. These isolates were screened and then subjected to a taxonomic study of their soluble protein profiles using

SDS-PAGE. These isolates were then compared with authentic reference strains to determine their identity. The results are presented in Chapter 3 of this thesis.

The second objective was to subject unidentified clusters resulting from the SDS-PAGE analyses, to a full polyphasic taxonomic study involving the latest taxonomic techniques such as the determination of 16S rRNA sequences, DNA-DNA hybridisation, fatty acid methyl ester analyses and comparative phenotypic analyses. As a result of these procedures two new *Chryseobacterium* species have been proposed and characterised. These results will be discussed in Chapters 4 and 5.

The third objective was to identify possible sources of flavobacterial contaminants in a broiler-processing factory. Research by other workers done on food, especially red meat, poultry and fish, have revealed that flavobacterial strains are basically always present during food spoilage although their source of contamination is not always clear (García-López *et al.,* 1998, 1999; Chattopadhyay, 1999; Hang'ombe *et al.*, 1999; Nychas & Drosinos, 1999; Forsythe, 2000). These results will be presented in Chapter 6.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The group of Gram-negative bacteria collectively known as the flavobacteria have, overtime, been assigned various roles. In the clinical environment some genera and species have been found to be pathogenic to humans and animals. In the food environment they have increasingly become associated with the spoilage of food and food products. The taxonomy of the flavobacteria has, however, undergone many changes since its inception in 1923 (Hendrie *et al.*, 1969; McMeekin & Shewan, 1978; Holmes *et al.*, 1984; Bernardet *et al.*, 1996).

During the past decade, *Flavobacteriaceae* has emerged as a family for a variety of Gram-negative yellow-pigmented rods and *Flavobacterium* has become only one of several genera in this family (Bernardet *et al.*, 1996). Many of the *Flavobacterium* species that were associated with food spoilage and pathogenicity in the past, have now been grouped into other genera, such as *Bergeyella*, *Chryseobacterium, Empedobacter, Myroides* and *Weeksella*, in the *Flavobacteriaceae* family (Holmes, 1992; Hugo & Jooste, 2003). Due to this fairly new reclassification, literature with regard to food spoilage still refers to psychrotrophic bacteria of this group involved in spoilage as *Flavobacterium* or flavobacteria or CDC Group IIb organisms.

2.2 Taxonomy of the *Flavobacteriaceae*

2.2.1 History

In the first edition of *Bergey's Manual of Determinative Bacteriology* (Bergey *et al.*, 1923), the genus *Flavobacterium* consisted of 46 yellow-pigmented, mainly Gram-negative, species. In the fifth edition published in 1939 (Bergey *et al.*, 1939), the polar flagellates were removed and in the 1948 edition the genus was grouped together with *Alcaligenes* and *Achromobacter* in the family *Achromobacteriaceae*

(Bergey & Breed, 1948). In the seventh edition (1957), the Gram-positive strains were removed and only 26 species remained (Weeks & Breed, 1957). These were reduced to 12 species and grouped into two sections in 1974 in the eighth edition (Weeks, 1974). The two sections, containing six species each, were divided according to the mole percentage guanine and cytosine content (% G + C). Group I had a low % G + C of 26 to 40 and Group II a high % G + C of 63 to 70 (Weeks, 1974). In the 1984 edition only the seven low DNA base ratio strains remained (Holmes *et al.*, 1984). In this edition*, Flavobacterium* was described as consisting of Gram-negative, yellow, non-motile, aerobic rods usually growing at temperatures from 5 °C to 30 °C and isolated from environmental and clinical sources. Some clinical strains like [*Flav*]*. meningosepticum*, could grow at 37 °C (Holmes *et al.*, 1984). With the introduction of a chemotaxonomic approach and more molecular microbiological techniques such as rRNA sequencing, it was possible to refine the differentiation between closely related genera as predicted by Holmes *et al.* (1984). They observed that chromosomal DNA characteristics made more accurate differentiation between various species possible (Holmes *et al.*, 1984).

2.2.2 Current Taxonomy

The family *Flavobacteriaceae*, as it stands today, is a member of the domain *Bacteria.* Figure 2.1 gives an outline diagram of the latter domain (Bernardet *et al.*, 2002).

Jooste (1985) had earlier suggested that the genus *Flavobacterium* should be accommodated in a new family *Flavobacteriaceae*. This suggestion was accepted by Reichenbach (1989) and the family was grouped under the order Cytophagales. This grouping provided the genus *Flavobacterium* with an affiliation and provided more perspective in understanding the relationship between *Flavobacterium* and *Cytophaga*.

Domain	Family	Description
	Bacteroidaceae	Gram-negative anaerobic rods
	Cytophagaceae	Anaerobes
Bacteria	*Flavobacteriaceae*	'Flavobacter-Bacteroides' phylum *Flavobacterium-Cytophaga* complex rRNA super family V *Cytophaga-Flavobacterium-Bacteroides* complex
	Sphingobacteriaceae	Closely related soil and clinical organisms
	Spirosomaceae	4 genera, distantly related
	Unaffiliated taxa	

Fig. 2.1 An outline diagram of the domain *Bacteria* in which the *Flavobacteriaceae* family are grouped

The family *Flavobacteriaceae* was validated in 1992 (Reichenbach, 1992) and the following genera were included *Bergeyella, Capnocytophaga, Chryseobacterium, Empedobacter, Flavobacterium, Ornithobacterium, Riemerella, Weeksella* and *Myroides.* Since then, other genera were added: *Gelidibacter* and *Psychroserpens* (Bowman *et al.*, 1997), *Polaribacter* (Gosink *et al.*, 1998), *Psychroflexus* (Bowman *et al.*, 1998) and *Salegentibacter* (McCammon & Bowman, 2000), *Cellulophaga* (Johansen *et al.*, 1999) and *Coenonia* (Vandamme *et al.*, 1999), *Tenacibaculum* (Suzuki *et al.*, 2001) and *Zobellia* (Barbeyron *et al.*, 2001) and in 2002 the family consisted of these 18 well-defined genera and two unaffiliated taxa (Bernardet *et al.*,

2002). New genera included in the family since 2002, were *Arenibacter* (Ivanova *et al.*, 2001), *Aequorivita* (Bowman & Nichols, 2002), *Mesonia* (Nedashkovskaya *et al.,* 2003a), *Vitellibacter* (Nedashkovskaya *et al.,* 2003b), *Algibacter* (Nedashkovskaya *et al.,* 2004c), *Formosa* (Ivanova *et al.*, 2004), *Gillisia* (Van Trappen *et al.*, 2004), *Kordia* (Sohn *et al.*, 2004), *Maribacter* (Nedashkovskaya *et al.,* 2004a), *Robiginitalea* (Cho & Giovannoni, 2004) and *Ulvibacter* (Nedashkovskaya *et al.,* 2004b; Bernardet, 2004). Table 2.1 contains a summary of the genera in the family with the type species or described species of each and the source of isolation.

The basic description of genera of the family *Flavobacteriaceae* is that they are Gram-negative, short to moderately long, non-spore forming rods. Their dimensions are 0.3 to 0.6 μm wide and 1 to 10 μm long. Cells of *Flavobacterium, Gelidibacter, Psychroserpens* and *Tenacibaculum* become spherical to coccoid in older cultures. The genera are oxidase and catalase positive. They are non-motile, but some genera show gliding motility (*Capnocytophaga, Cellulophaga, Gelidibacter, Flavobacterium, Tenacibaculum* and strains of *Zobellia* and *Psychroflexus torquis*). Most of them are aerobic, but *Capnocytophaga, Coenonia, Ornithobacterium* and *Riemerella* strains are microaerophilic to anaerobic. The optimum growth temperature ranges from 25 to 35 °C, but *Gelidibacter, Polaribacter, Psychroflexus, Psychroserpens* and *Salegentibacter* strains are psychrophilic and certain strains of *Flavobacterium* are psychrotolerant. Their ability to grow in different salt concentrations varies considerably. The majority of strains are pigmented by carotenoid and/or flexirubin pigments, although, strains of *Bergeyella, Coenonia, Ornithobacterium* and *Weeksella* are non-pigmented. The DNA base composition varies from 27 to 44 mol % G + C (guanine and cytosine). All genera in the family *Flavobacteriaceae* exhibit menaquinone 6 as the only or main quinone whereas other related families exhibit menaquinone 7 as main quinone (Bernardet *et al.*, 2002).

Table 2.1 Genera in the family *Flavobacteriaceae* and recorded sources of isolation

Genus	Type / descriptive species	Source
Flavobacterium[1] (type genus)	***Flavobacterium aquatile***	Fresh and salt water, fish, soil
Aequorivita[4]	*Aequorivita lipolytica*	Antarctic seawater, sea-ice assemblages, quartz stone
Algibacter[7]	*Algibacter lectus*	Green algae, sea of Japan
Arenibacter[2]	*Arenibacter latericius*	Sandy sediments, South China sea
Bergeyella[1]	*Bergeyella zoohelcum*	Clinical - human, dairy processing environment
Capnocytophaga[1]	*Capnocytophaga ochracea*	Clinical - human
Cellulophaga[3]	*Cellulophaga lytica*	Marine environment
Chryseobacterium[1]	*Chryseobacterium gleum*	Clinical - human & fish, water, marine environment, fish, milk
Coenonia[3]	*Coenonia anatina*	Poultry
Empedobacter[1]	*Empedobacter brevis*	Clinical – human
Formosa[8]	*Formosa algae*	Thallus of brown algae
Gelidibacter[3]	*Gelidibacter algens*	Sea ice
Gillisia[9]	*Gillisia limnaea*	Microbial mats, Lake Fryxell, Antarctica
Kordia[10]	*Kordia algicida*	Red tide
Maribacter[11]	*Maribacter sedimenticola*	Marine habitats, South China sea

Table 2.1 continued

Mesonia[5]	*Mesonia algae*	Green algae, South China sea
Myroides[1]	*Myroides odoratus*	Clinical – human
Ornithobacterium[1]	*Ornithobacterium rhinotracheale*	Clinical – poultry
Polaribacter[3]	*Polaribacter filamentus*	Fresh and salt water
Psychroflexus[3]	*Psychroflexus torquis*	Salt water
Psychroserpens[3]	*Psychroserpens burtonensis*	Salt water
Riemerella[1]	*Riemerella anatipestifer*	Clinical – poultry
Robiginitalea[12]	*Robiginitalea biformata*	Marine habitat
Salegentibacter[3]	*Salegentibacter salegens*	Organic water
Tenacibaculum[3]	*Tenacibaculum maritimum*	Marine environment, coast of Japan
Ulvibacter[13]	*Ulvibacter litoralis*	Green algae, South China sea
Vitellibacter[6]	*Vitellibacter vladivostokensis*	Holothurian, sea of Japan
Weeksella[1]	*Weeksella virosa*	Clinical - human, dairy processing environment
Zobellia[3]	*Zobellia galactanivorans*	Marine environment

1, Jooste & Hugo (1999); 2, Ivanova *et al.* (2001); 3, Bernardet *et al.* (2002); 4, Bowman & Nichols (2002); 5, Nedashkovskaya *et al.* (2003a); 6, Nedashkovskaya *et al.* (2003b); 7, Nedashkovskaya *et al.* (2004c); 8, Ivanova *et al.* (2004); 9, Van Trappen *et al.* (2004); 10, Sohn *et al.* (2004); 11, Nedashkovskaya *et al.* (2004a); 12, Cho & Giovannoni (2004); 13, Nedashkovskaya *et al.* (2004b).

2.3 The genus *Chryseobacterium*

2.3.1 Ecology and Characteristics

The genus *Chryseobacterium* was proposed by Vandamme *et al.* (1994). Species of this genus are widely distributed in environmental sources, like water and soil and in clinical sources (Vandamme *et al.*, 1994).

The genus *Chryseobacterium* is described as consisting of very small rods, 0.5 μm wide and 1 to 3 μm long. Colonies are usually yellow to orange in colour as they contain the pigment flexirubin, although *C. meningosepticum* (now *Elizabethkingia meningoseptica*) colonies may be colourless to very weak yellow. The DNA base composition ranges between 33 to 38 mol % G + C. Other characteristics include hydrolysis of esculin, predominance of branched chain fatty acids, absence of sphingophospholipids and the production of menaquinone 6 as the only respiratory quinone (Vandamme *et al.*, 1994). Strong proteolytic activity occurs, that may make them potential meat spoilers. They show resistance to a range of antimicrobials (Vandamme *et al.*, 1994; Kirby *et al.*, 2004).

2.3.2 Taxonomy of *Chryseobacterium*

The genus *Chryseobacterium* was proposed in the mid nineties by Vandamme *et al.* (1994) and several erstwhile "*Flavobacterium*" strains have been included in this genus (Bernardet *et al.*, 1996, 2002). Several flavobacteria, formerly referred to as the *Flavobacterium* CDC Group IIb or Group A of Holmes (1992), were renamed and grouped in the *Chryseobacterium* genus on the basis of fitting into a tight rRNA cluster. *Flavobacterium* species that have been renamed include: *Chryseobacterium* [*Flav.*] *indologenes, Chryseobacterium* [*Flav.*] *gleum, Chryseobacterium* [*Flav.*] *indoltheticum*, *Chryseobacterium* [*Flav.*] *balustinum, Chryseobacterium* [*Flav.*] *meningosepticum* with *C. gleum* as type species (Bernardet *et al.*, 2002). The fish pathogen, *C.* [*Flav.*] *scophthalmum,* was also included in this genus in 1994 (Mudarris *et al.,* 1994). One strain namely [*Flav.*] *brevis,* occupied another position in the rRNA cluster, and has been regrouped

and renamed *Empedobacter brevis* (Vandamme *et al.*, 1994; Bernardet *et al.*, 2002).

Previous studies in the Department of Food Science, UFS, have shown that yellow pigmented flavobacterial strains from the food environment (raw milk) were actually members of the genus *Chryseobacterium.* Some of these studies lead to the description of *Chryseobacterium joostei* isolated from raw milk (Hugo, 1997; Hugo *et al.*, 2003). Table 2.2 contains a summary of the species of the genus *Chryseobacterium* and the recorded original source of isolation.

Since the publication of Bernardet *et al.* (2002), new species have been validated. *Chryseobacterium defluvii,* isolated from sewage water (Kämpfer *et al.*, 2003) and *E. miricola,* isolated from condensation water in a Russian space station (Li *et al.*, 2003) were also introduced to the study. The latest validated species of this genus are *C. formosense* (Young *et al.*, 2005), *C. daecheongense* (Kim *et al.*, 2005a) and *C. taichungense* (Shen *et al.*, 2005). The species validated in 2005 were not included in this study. One strain, '*C. proteolyticum'* was described by Yamaguchi & Yokoe (2000) but has not been validly published (Bernardet *et al.*, 2002). Mean while, it was also proposed that two *Chryseobacterium* species, *C. meningosepticum* and *C. miricola,* are renamed in a new genus, *Elizabethkingia* (Kim *et al.*, 2005b). Of the currently validated species of *Chryseobacterium,* only *C. balustinum, C. gleum, C. indologenes and C. joostei* are often associated with food (Hugo & Jooste, 2003).

Table 2. 2 Species in the genus *Chryseobacterium*, recorded sources and dates of isolation

***Chryseobacterium* species**	**Original source of isolation**	**Date**
C. balustinum [1]	Blood of fresh water fish, France	1959
C. daecheongense[7]	Sediment, freshwater lake	described 2005
C. defluvii [4]	Wastewater	described 2003
C. formosense[6]	Rhizoshere of garden lettuce, Taiwan	described 2004
C. gleum [1] (type species)	Human vaginal swab, UK	1979
C. indologenes [1]	Human trachea autopsy	1958
C. indoltheticum [1]	Marine mud	described 1951
C. joostei [2]	Raw cow's milk, RSA	1981
'C. proteolyticum' [3]	Rice field soil, Japan	described 2000
C. scophthalmum [1]	Gills of marine fish, UK	1987
C. taichungense[8]	Soil, Taiwan	described 2005
Elizabethkingia [Chrys.] meningoseptica [1]	Human cerebrospinal fluid, USA	1949
Elizabethkingia [Chrys.] miricola [5]	Space station, Russia	1997

1, Bernardet *et al.* (2002); 2, Hugo *et al.* (2003); 3, Yamaguchi & Yokoe (2000); 4, Kämpfer *et al.* (2003); 5, Li *et al.* (2003); 6, Young *et al.* (2005); 7, Kim *et al.* (2005a); 8, Shen *et al.* (2005).

2.3.3 Potential Pathogenicity

Flavobacteria are not usually pathogenic, although some strains show a low degree of pathogenicity. Other strains may be opportunistic pathogens, where they pose a risk to immuno-compromised patients (Sheridan *et al.,* 1993). Some recent studies indicated that bacteria isolated under normal conditions on plate count agar, tend to be not as harmless as generally thought and the genus *Chryseobacterium* has been included as one of the frequently isolated heterotrophic species with potential pathogenic features (Pavlov *et al.*, 2004).

Several cases of infection in which *Chryseobacterium* species (or reclassified species) were implicated, have been reported and these include clinical cases of lower respiratory tract infections in immuno-compromised patients. Burn wound infections and fatalities in England, 2001 were caused by unidentified *Chryseobacterium* species and in two cases the source of contamination was untreated water (Fraser & Jorgensen, 1997; Kienzle *et al.*, 2001). Wound sepsis by *Elizabethkingia meningoseptica* as causative agent in burn wounds of two pediatric patients showed resistance to antibiotics (Sheridan *et al.,* 1993), while Bloch *et al.* (1997) reported on six cases of *E. meningoseptica* pneumoniae in immuno-compromised patients. *Chryseobacterium indologenes* was implicated in conjunctivitis in Switzerland and it is believed that water systems in the hospital were responsible for contamination (Lu & Chan, 1997). *E. meningoseptica* was found to be responsible for cases of meningitis and was also isolated from an infant with unresolved diarrhoeal disease (Springer & Johnson, 1999). In Turkey, in the neonatal intensive care unit (in 2001), an infant died and three others developed *E. meningoseptica* sepsis. Investigation found that the organism had been transferred through an intravenous lipid solution given to the infants and *E. meningoseptica* was isolated from the lipid stock solution bottle (Gungor *et al.*, 2003).

Pan *et al.* (2000) indicated that enzyme activity and especially protease activity was significantly higher in invasive isolates of *C. indologenes* compared to the non-invasive isolates. The invasive isolates were recovered from blood samples while the non-invasive isolates were recovered from other clinical samples from the National Taiwan University Hospital (Pan *et al.*, 2000). *Chryseobacterium scophthalmum* was associated with diseased turbot gills (Mudarris *et al.,* 1994).

Flavobacteria in general, show resistance to several antibiotics and it was advised that *Chryseobacterium* isolates from clinical infections should undergo susceptibility tests to find the most suitable antibiotic for therapy as they may be susceptible to antibiotics usually used for Gram-positive infections (Chang *et al.,* 1997; Fraser & Jorgensen, 1997; Kirby *et al.*, 2004).

2.4 Flavobacteria in the food environment

Giraffa & Neviani (2001) claimed that food is an unexplored source of bacterial diversity and that several organisms isolated from food were incorrectly classified. Apart from the fact that the classification of many Gram-negative organisms has changed, the diversity referred to in food is well recognised. This limits the appropriate literature on members of the family *Flavobacteriaceae* and specifically *Chryseobacterium*, that have previously been isolated from food sources. *Chryseobacterium joostei* (Hugo *et al.*, 2003), is an exception having been isolated from milk.

Available literature has mentioned the role of flavobacteria together with the pseudomonads in food spoilage (García-López *et al.,* 1998*,* 1999; Forsythe, 2000; Jay, 2000, Gram *et al.*, 2002). Undesirable flavours and odours, possible slime production and/or toxic metabolic end products are detrimental and apart from an economical loss to industry and consumers, also have a possible health impact on consumers. The argument that raw meat will be consumed cooked and that the heat treatment during cooking will kill all organisms, is only valid to a certain extent. Severity of the heat treatment and the number and type of organisms will determine the amount of the metabolic end-products and the resultant quality of the product. Toxic chemical products already formed, may not always easily be eliminated. Human response to such chemical compounds is not always clearly understood. Exposure to low concentrations of toxicants may lead to allergic reactions or even anaphylactic reactions. Immuno-compromised persons, such as people with HIV positive status and TB patients, malnutritioned children and the elderly are at risk (Nicklin *et al.*, 1999). High numbers of bacteria ingested will cause a health risk to any person, but especially immuno-compromised persons. Food products of inferior quality will pose a health risk to consumers.

Even if the spoilage bacteria are not pathogenic *per sé*, changes in the biochemical status of stored food due to deterioration by such bacteria, may make conditions favourable for other bacteria or even pathogens to grow in. Proteolysis and lipolysis are also responsible for degradation of the nutritional value of food and will further have a health impact on the community. Flavobacteria have been

associated with spoilage of food, but the incidence and role of flavobacteria in food deterioration is difficult to obtain, mainly due to the history of faulty classification or reclassification of these organisms. They are, however, accepted as common contaminants of protein rich foods and under refrigerated storage, they are in competition with the pseudomonads (García-López *et al.*, 1999). Sources of contamination exist throughout the production process and contributing factors are environmental sources such as hides and feathers, processing water throughout the process, intestinal contamination during the slaughter process, food handlers and vectors such as insects and rodents. It is generally believed that pathogens are normally introduced to the food via contact with intestinal secretions or food handlers and that spoilage organisms contaminate the food via environmental sources such as dust and food contact surfaces (Forsythe, 2000; Jay, 2000). In a study by Dugas *et al.* (2001), a *Chryseobacterium* strain was isolated from the gut of an American cockroach. This information may be valuable in identifying sources of contamination, especially through vectors (Forsythe, 2000).

2.4.1 Perishable food products

Flavobacteria are frequently found in the dairy processing environment and they are responsible for several defects in dairy products (Jooste & Hugo, 1999). Several *Chryseobacterium* species are associated with spoilage of dairy products during cold storage and include *C. balustinum, C. gleum* and *C. joostei* (Bernardet *et al.*, 2002). In milk they produce heat resistant proteolytic and lipolytic enzymes responsible for off-flavours in pasteurised milk and cream, surface taint in butter and thinning of creamed rice. It also is responsible for reduction in cheddar cheese yield (García-López *et al.*, 1999; Jooste & Hugo, 1999; Bernardet *et al.*, 2002). In eggs they are responsible for coagulation of liquid egg and the production of faecal, fishy, sulphuric and ammonia odours (García-López *et al.*, 1999).

Raw poultry, fish and meat are highly perishable products that are very susceptible to spoilage by several microorganisms. The type of organism developing on raw fish and meat will be determined by intrinsic and extrinsic parameters. Intrinsic parameters include aspects such as water activity, oxygen, available nutrients and

pH. The pH range of red meat and poultry is 5.4 to 6.4 while it is slightly higher for fish namely, 6.6 to 6.8. The most important extrinsic factors contributing to a specific bacterial population are the storage temperature and the atmospheric composition. Spoilage is caused when chemical changes and the production of metabolites, by microorganisms, cause off flavours to develop (Forsythe, 2000). Spoilage of protein rich foods results when ammonia, hydrogen sulphide, indole and other amines are formed with a consequent increase in pH. Two diamines, cadaverine and putrescine, are spoilage indicators and are produced by decarboxylation of lysine, ornithine and arginine (Jay, 2000).

2.4.2 Poultry

Usually poultry are associated with pathogens like *Salmonella* and *Campylobacter* (Banwart, 1989), but large numbers of other bacteria, often associated with spoilage, are found on poultry carcasses (Forsythe, 2000; Jay, 2000). Apart from the Gram-positive strains, several Gram-negative strains are present on poultry, including *Pseudomonas* spp., *Flavobacterium* spp., *Alcaligenes* spp., *Klebsiella* spp. and *E. coli* (Hang'ombe *et al.*, 1999; Hinton *et al.*, 2004; Vazgecer *et al.*, 2004). Hinton *et al.* (2004), also found that there was a significant increase of psychrotrophic spoilage bacteria during processing due to cross-contamination and that these bacteria were responsible for spoilage of poultry during refrigerated storage. The source of origin of these organisms, may be from the poultry itself or from the abattoir environment (Hang'ombe *et al.*, 1999). Mai & Conner (2001) found that the incidence of *Pseudomonas* and *Flavobacterium* on chicken carcasses were 17 % and 16 % respectively. Nychas & Drosinos (1999), found that the incidence of flavobacteria on poultry is much higher than on fresh red meat.

2.4.3 Fish

On cold-water marine fish, the Gram-negative bacteria responsible for spoilage are *Achromobacter, Flavobacterium* and *Pseudomonas,* and these genera dominate the surface counts (Chattopadhyay, 1999). *Chryseobacterium balustinum* was found to be associated with the production of yellow slime on

halibut (Bernardet *et al*., 2005). Raw fish is rich in trimethylamine oxide (TMAO) which plays an important role in microbial degradation of freshly caught fish. Although autolytic processes are responsible for many catabolic biochemical reactions in fresh fish, microorganisms also make a major contribution to the organoleptic deterioration of this commodity (Leisner & Gram, 1999). Metabolites produced by spoilage organisms in fish are similar to the products formed during spoilage of poultry and meat. These catabolites include ammonia, amines and sulphides. One metabolite namely, trimethylamine (TMA) is distinctive in fish spoilage and is formed during anaerobic respiration of trimethylamine oxide. Trimethylamine has a ammonia-like “fishy” odour (Gram & Dalgaard, 2002). Other typical fish spoilage odours are fruity, pungent and musty and are mainly produced by Gram-negative bacteria such as the pseudomonads and flavobacteria (Engelbrecht *et al*., 1996). Psychrotolerant Gram-negative bacteria are responsible for spoilage of fish during cold storage. Apart from unacceptable off-flavours produced by micro-organisms, discoloration and slime production will also contribute to spoilage of fish. The identification of specific spoilage organisms (SSO) will contribute to a better understanding of fish spoilage since spoilage organisms differ from one seafood to another (Gram & Dalgaard, 2002).

2.4.4 Red Meat Spoilage

Spoilage of raw red meat will result in off-odours, possible slime production, discoloration of a specific area and undesirable flavours due to metabolic end-products formed. In a study by Labadie (1999), it was stated that the reasons for the presence of specific spoilage bacteria on meat were not clear and little information on bacterial protein metabolism is available. It is known however, that they are responsible for discoloration and off-odours. Metabolites 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 metabolism (Banwart, 1989). Off or malodours can be described as fishy, foul, sulphuric and ammonia-like (Nychas & Drosinos, 1999). Jay *et al.* (2003), found that Gram-negative strains increased with time and *Pseudomonas* dominated the spoilage flora of refrigerated ground beef.

Chryseobacterium gleum and *C. indologenes* are often present on raw meat (Bernardet *et al.*, 2005).

2.5 The Polyphasic taxonomic approach as tool in bacterial classification

Classification of prokaryotic taxa since 2001 has been based on phylogenetic features rather than on the phenotypic features used previously (Cole *et al.,* 2003). However, any species classified according to phylogenetic similarities must show phenotypic consistency. The introduction of a polyphasic taxonomic approach integrates phenotypic, genotypic and phylogenetic information to form a solid basis for bacterial taxonomy (Vandamme *et al.*, 1992). Janda & Abbott (2002) emphasized that the use of single techniques or sequence analysis of only one or two strains could yield to misidentification. Combinations of phenotypic and genotypic testing methods give the most reliable results for identification and have proved to be a valuable newer approach to bacterial taxonomy (Vandamme *et al.*, 1996; Janda & Abbott, 2002).

Chemotaxonomy gives valuable and reliable information on chemical characteristics for classification and identification purposes. This approach (analysis of the chromosomal DNA, ribosomal RNA, and fatty acids) can distinguish accurately between genera and species. Ribosomal RNA (rRNA) has a highly conserved structure that is well documented to give reliable phylogenetic levels on phylogenetic trees. The use of the rRNA sequence is currently an important tool in bacterial identification (Ludwig *et al.*, 1998). DNA-DNA hybridisation is a labour intensive and complex method and only a few laboratories, are equipped to perform these analyses. In order to compare data properly, well-standardised methods must be used, otherwise results can easily be misinterpreted.

2.5.1 SDS-PAGE and FAME analysis

Whole cell protein finger prints with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and fatty acid composition of strains by means of

fatty acid methyl ester analysis (FAME), can distinguish groups of bacteria up to genus level (Austin & Priest, 1986).

2.5.1.1 Electrophoresis of cellular proteins

Total cellular proteins produced under standardised conditions, produce complex, but reproducible and representative fingerprints of bacterial strains. These fingerprints are a useful tool in bacterial classification (Austin & Priest, 1986; Pot *et al.*, 1994). Extracted proteins are subjected to electrophoresis on polyacrylamide gels, protein bands are compared and patterns are usually similar for similar species. Strains that show at least 70% DNA binding values tend to display similar protein fingerprints, with only minor differences (Pot *et al.*, 1994).

The basic principle of these comparisons is the recording of the different protein concentration patterns using a densitometer. During convertion of the data, protein patterns are standardised, normalised and optimised and results of recorded data are represented in a dendrogram, indicating similar protein group patterns represented as clusters (Pot *et al.*, 1994).

2.5.1.2 Fatty acid methyl analysis (FAME)

Long chain fatty acids, as main constituents of the lipids present in bacterial cells, are frequently used in bacterial identification and provide useful information under standardised conditions and processes (Vandamme *et al.*, 1996). The fatty acid composition of the plasma membrane can be used to identify and classify bacteria and is done according to the hydroxy-, methyl- and cyclopropane content of the fatty acids in the membrane (Austin & Priest, 1986). Extraction of fatty acids consists of a few steps namely the saponification process after cells have been harvested, methylation to improve the volatility of the fatty acids in the column and extraction of fatty acids. With gas chromatography, fatty acids are separated and with the aid of commercial identification systems, comparisons and clustering are done (Vandamme *et al.*, 1992).

2.5.2 Molecular Genetic Techniques

Introduction of molecular genetic techniques to microbiology has refined taxonomy, since these methods provide information of the genetic structure of an organism. These techniques applied to ecological samples indicated a new perspective to microbial diversity and emerging organisms previously not culturable as well as detection and identification of organisms isolated from complex ecosystems (Hunter-Cevera, 1998; Giraffa & Neviani, 2001). The first report on specific DNA amplification with PCR was published in 1985 and since then, modifications of the basic method and its practical applications have increased tremendously (Erlich, 1992).

2.5.2.1 rRNA

Ribosomal RNA is present in all bacteria and specific segments, especially the 16S subunit, is highly conserved and contributes to comparisons between closely related organisms (genus level). Oligonucleotides on the 16S molecule are compared with DNA:rRNA hybridisation to determine the sequence of the tested strain against a reference strain (Austin & Priest, 1986; Woese, 1987).

Ribosomal RNA genes have conserved characteristics and amplified rDNA restriction analysis gives species-specific patterns and is, therefore, a valuable tool in genotypic analysis (Vandamme *et al*., 1996). The use of a universal bacterial primer pair during PCR amplification gives valuable information when an unknown bacterial strain is introduced and a single universal 16SrRNA primer pair allows species specific identification of the bacteria when introduced in a nucleotide sequence database (McCabe *et al.,* 1999). This method gives the relationship between the unknown or test strains and the reference strains in a genus. As phenotypic characterization often lacks the ability to clearly distinguish between different species, 16S ribosomal DNA, that is rDNA based, is a useful alternative especially when several bacterial isolates need to be classified (Drancourt *et al*., 2000).

The basic principle of 16S rRNA sequencing implies PCR amplification and purification of prepared DNA. Heat stable *Taq* (from *Thermus aquaticus*) polymerase in the presence of specific primers synthesises complementary strands of DNA. Synthesis is achieved by several cycles of heating, to separate the DNA strands, followed by cooling, when primers will anneal to the complementary sequence. After several cycles, the desired amount of amplification will be obtained (Taylor, 1991; Erlich, 1992). Introduction of a low percentage of magnesium chloride solution to the primer mix, improves PCR specificity and yield as Mg^{2+} quantitatively binds with the deoxynucleotide triphosphates (dNTP) (Erlich, 1992). Cobb & Clarkson (1994) demonstrated that modification in the dNTP concentrations needs adjustments in the $MgCl_2$ concentration, as excessive amounts of Mg^{2+} will lead to the accumulation of non-specific amplification products, and an insufficient Mg^{2+} concentration reduces the product yield. Alteration in the $MgCl_2$ concentration is seen as an easy way to promote amplification, rather than changes to the annealing temperatures (Cobb & Clarkson, 1994). The sequence reactions with conserved primers sequence the entire 16S rRNA in both directions.

2.5.2.2 Analysis of chromosomal DNA: DNA-DNA hybridisation

Purified and concentrated DNA, free from any traces of protein, RNA or carbohydrates from the bacterial cells is subjected to the determination (estimation) of the nucleotide composition. The amount of guanine and cytosine (mol % G + C) is specific for each species. The melting point (T_m) and mol % G + C are linearly related and yields information on the temperature at which the two strands of DNA are separated. High-molecular-weight DNA is obtained after several steps of denaturation, purification, washing and drying processes. The DNA:DNA duplex structure is specific for species. A hybridisation relation of more than 70 % and a melting point (T_m) of less than 5 °C are indicative of the same species (Austin & Priest, 1986; Jay, 2000). The basic technique for DNA hybridisation was described by Ezaki *et al.* (1989), and is still used. This method measures the renaturation rates of deoxyribonucleic acid to determine the genetic relationship between strains. It is done by the hybridisation of immobilised reference DNA with a labelled probe DNA. In a comparative study, Goris *et al.*,

(1998) showed that the microplate DNA-DNA hybridisation method of Ezaki *et al.* (1989) labelled with photobiotin, is a fast and reliable method for taxonomical studies compared to the time consuming classical enzymatic labelling method.

2.5.3 Phenotypic characteristics

The description of phenotypic characteristics of bacteria is an important tool and should be used in conjunction with above-mentioned molecular techniques. Basic identification should be possible using a general description by conventional methods. These methods include basic growth parameters such as growth at different temperatures, under aerobic or anaerobic conditions, the need for specific nutrients and the ability to produce specific metabolites. Production of enzymes will always give valuable information on potential spoilage abilities (*e.g.* proteolysis and lipolysis) while production of gas, metabolites containing alcoholic compounds and indole will contribute to certain flavour compounds (Banwart, 1989).

The majority of genera in the family *Flavobacteriaceae* produce a pigment in colonies on agar media ranging from cream and/or pale yellow to a bright orange yellow depending on the media and incubation temperature/time. However, standardisation on specific methodologies for description characteristics are important for inter-laboratory comparisons of data. Methods described in the proposed minimal standards should be followed (Bernardet *et al.,* 2002).

Figure 2.2 indicates a scheme for the basic identification of culturable bacteria. Valuable information can be obtained from microscopic examination with different staining techniques and biochemical reactions and in most cases it will be possible to differentiate between different genera and existing species. In order to effectively distinguish between different, but closely related species, molecular methods are necessary (Nicklin *et al.*, 1999). This statement is specifically true of a newly emerged strain that has been isolated.

A species by definition “consists of strains of common origin, more similar to each other than to any other strain”. Thus will apply to strains sharing DNA-DNA pairing values of more than 70 % and 16S rRNA sequence similarities of more than 97 %

(Dijkshoorn *et al.*, 2000). In order to complete a taxonomical study, all phenotypic and genotypic information must be integrated and strains can then be classified within existing taxonomical structures.

2.6 Conclusion

The polyphasic taxonomic approach combines phenotypic and genomic characteristics that are necessary to classify existing or describing new organisms. Introduction of molecular techniques in microbiology was a scientific development in bacterial taxonomy that enabled microbiologists to classify bacteria precisely to the species level according to genotypic characteristics of different closely related strains. However, the importance of phenotypic characteristics should never be neglected, but it should be emphasized that standardisation of methods in description of phenotypic characteristics are extremely important in order to make presumptive identification. The availability of genomic information leads to changes in current taxonomical structures and research is necessary to gain and extend the knowledge.

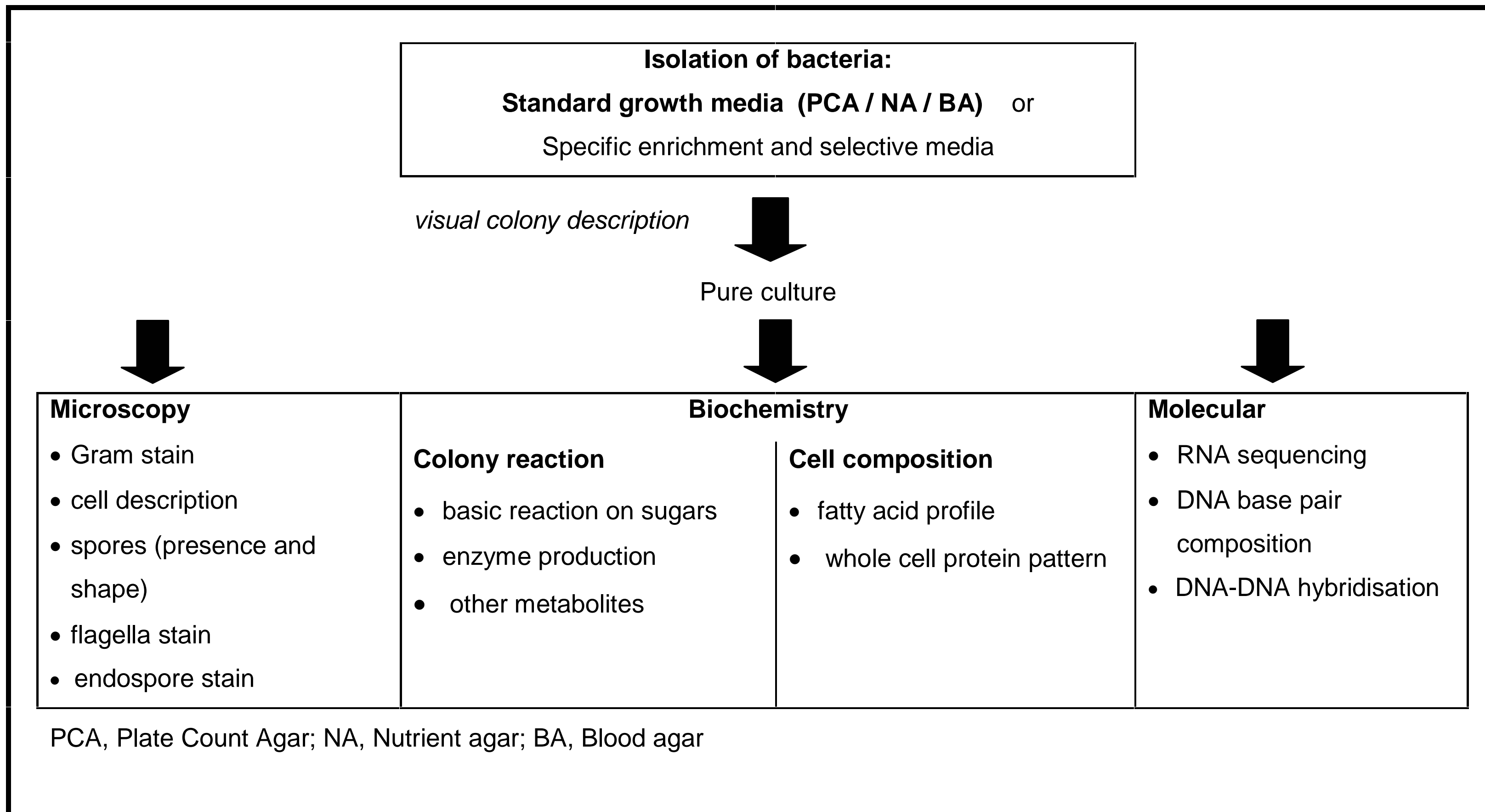

Fig. 2.2 Basic scheme for bacterial identification

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CHAPTER 3

ISOLATION OF MEMBERS OF THE FAMILY *FLAVOBACTERIACEAE* FROM RAW POULTRY, FISH AND MEAT

This article will be submitted for publication in the International Journal of Food Microbiology

Abstract

Changes in the taxonomy of the yellow Gram-negative strains during the past decade, are accountable for the fact that many organisms, previously known as *Flavobacterium*, now belong to different genera of the larger family *Flavobacteriaceae*. Some of these strains, especially *Chryseobacterium, Empedobacter* and *Myroides* were previously associated with food spoilage under refrigerated conditions. Several yellow-pigmented bacterial strains from raw poultry, fish and meat were isolated and subjected to a screening test for *Chryseobacterium, Empedobacter* and *Myroides* strains and then subjected to SDS-PAGE analyses for a preliminary identification. Results indicated that the majority of Gram-negative yellow-pigmented isolated were members of the genus *Chryseobacterium*. A few clusters from the SDS-PAGE dendrogram did not contain any known reference strains and could be regarded as possible new species.

3.1 Introduction

The high protein content of meat and fish give it a predictive perishable character and spoilage takes place even under refrigerated conditions. During slaughtering and processing practices, the so called “sterile” meat is introduced to several environmental and enteropathogenic organisms that are responsible for deterioration of the meat during refrigerated storage conditions (Forsythe, 2000). Psychrotrophic bacteria are able to grow under these conditions. Literature mainly refers to the pseudomonads as the most important group of psychrotrophs

responsible for deterioration (Forsythe, 2000; Jay, 2000). Another group of bacteria often mentioned are the micro-organisms with yellow-pigmented colonies, formerly and generally referred to as the flavobacteria (Hendrie *et al.*, 1969).

The taxonomy of the flavobacteria has changed drastically in the past decade and many organisms then known as *Flavobacterium*, now belong to the larger family *Flavobacteriaceae* but to different genera in this family (Vandamme *et al.*, 1994; Bernardet *et al.*, 2002). Most of the food spoilage flavobacteria, consequently, now belong to the genera *Chryseobacterium, Empedobacter* and *Myroides*.

The aim of this study will, therefore, be to isolate yellow-pigmented bacterial isolates from different meat products, perform a screening test for *Chryseobacterium, Empedobacter* and *Myroides* strains and then do a preliminary identification by grouping the isolates using SDS-PAGE.

3.2 Materials and Methods

3.2.1 Isolation and growth conditions of yellow-pigmented isolates

Chicken samples were obtained from a local chicken processing plant and consisted of neck skin samples taken from chicken carcasses at different points on the processing line. Meat samples were obtained from fresh cut beef samples sent for routine analysis to ARC-Irene Animal Production Institute (ARC-IAPI) and minced meat samples were obtained from a local butchery.

Serial dilutions of raw chicken and minced beef samples were plated on Plate count agar plates (Oxoid CM463), incubated at 4 °C for 24 hours and then 48 hours at 25 °C. Different yellow-pigmented colonies were picked from PCA plates and sub-cultured until pure colonies were obtained. Marine fish isolates were obtained from a previous study performed in 1996 in the Department of Food Science at the University of the Free State (Lingalo, 1997). These isolates originated from Cape marine fish. The reference strains used were acquired from the culture collection of the University of Ghent (LMG Bacteria Collection, Laboratory for Microbiology, University of Ghent, Belgium).

All the strains were maintained as freeze-dried cultures on filter paper discs after suspending the cells in a 10 % lactose skim milk medium (Britz & Kriel, 1973) and stored in screw capped tubes at –20 °C. Strains were reactivated in 10 ml Nutrient Broth (Oxoid CM67) and purity checked by streaking on Nutrient Agar (Oxoid CM3). Except for the initial incubation at 4 °C for 24 hours all other incubation was at 25 °C for 48 hours as prescribed by Hugo & Jooste (2003).

Table 3.1 Reference strains used in this study

Strain	Code
Chryseobacterium balustinum	LMG 8329 ^{T}
Chryseobacterium defluvii	LMG 22469 ^{T}
Chryseobacterium gleum	LMG 8334 ^{T}
Chryseobacterium indologenes	LMG 8337 ^{T}
Chryseobacterium indoltheticum	LMG 4025 ^{T}
Chryseobacterium joostei	LMG 18212 ^{T}
Elizabethkingia meningoseptica	LMG 12279 ^{T}
Elizabethkingia miricola	LMG 22470 ^{T}
Chryseobacterium scophthalmum	LMG 13028 ^{T}
Bergeyella zoohelcum	LMG 8351 ^{T}
Empedobacter brevis	LMG 4011 ^{T}
Flavobacterium aquatile	LMG 4008 ^{T}
Myroides odoratus	LMG 1233 ^{T}
Sphingobacterium spiritivorum	LMG 8347 ^{T}
Weeksella virosa	LMG 12995 ^{T}

LMG, Bacteria Collection, Laboratory for Microbiology, University of Ghent, Belgium.

3.2.2 Screening for *Chryseobacterium, Empedobacter* and *Myroides* strains

All yellow and orange-yellow colonies were streaked on Brilliant Green agar (Oxoid CM329) in order to eliminate any Gram-positive yellow-pigmented organisms. These plates were incubated for 48 hours at 25 °C. Yellow colonies on the red medium were subjected to a screening regime for *Chryseobacterium, Empedobacter* and *Myroides* strains as depicted in Table 3.2. The screening regime was based on the review by Bernardet *et al.* (2002).

Table 3.2 Screening regime with reactions specific for *Chryseobacterium, Empedobacter* and *Myroides* strains

Screening Test	Reaction
Gram stain	Gram negative, small bacilli*
Oxidase	Positive
Catalase	Positive
Flexirubin production	Positive
Motility	Negative

* Resembles coccobacilli under 100X oil immersion light microscopy.

3.2.3 SDS-PAGE of whole cell proteins

Whole cell proteins of 146 isolates and 13 reference organisms were extracted from a 24 hours old culture on Nutrient agar (Oxoid CM3) as prescribed by Pot *et al.* (1994). SDS-PAGE was then performed on these extracts according to Pot *et al.* (1994) with *Psychrobacter immobilis* used as control strain on each gel.

Electrophoresis was performed in a 1.5 mm separation gel slab (12 % total acrylamide with 2.67 % cross-linking), run vertically (35 mA constant current per gel at 15 °C) until the bromophenol blue tracking dye had migrated 10 cm from the

top of the separation gel. Detection of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (*r*) and UPGMA cluster analyses were performed as described by Pot *et al.* (1994) using the GELCOMPAR II software package (Applied Maths BVBA, 1998, Sint-Martens-Latem, Belgium).

3.3 Results and Discussion

3.3.1 Isolation of potential strains

A variety of different yellow-pigmented colonies were collected from the PCA plates. A summary of the number of samples obtained, the number of yellow-pigmented cultures isolated from these samples and the number of whole cell protein extracts exposed to SDS-PAGE, are given in Table 3.3. In the final dendrogram, only 127 isolated strains and 13 reference strains were presented, since a few strains died off during prolonged frozen storage and transport to Belgium.

The majority of yellow colonies isolated from red meat were Gram-positive. Brilliant Green agar was then introduced as part of the screening regime to eliminate Gram-positive yellow-pigmented colonies and therefore, the majority of isolates were obtained from chicken. Thus, the low number strains isolated from meat does not necessarily reflect their prevalence, although Nychas & Drosinos (1999) found that the incidence of flavobacteria on poultry is much higher than on fresh red meat. In this study the amount of yellow colonies per sample from the raw chicken carcasses varied considerably. These samples were obtained from chicken carcasses at different stages of processing namely, before and after the spin chilling process, during the spiral freezing process, during the manual and automatic processing line and from individual portions as well as from whole bird samples.

No deduction as to the significance of differences in the occurrence of yellow-pigmented colonies from the different areas could be made, since no statistical analyses were performed. According to a study by Mai & Conner (2001),

however, it was found that the incidence of *Pseudomonas* and *Flavobacterium* on chicken carcasses were 17 % and 16 % respectively. Chattopadhyay (1999) found that *Achromobacter, Flavobacterium* and *Pseudomonas* were the main groups of Gram-negative bacteria responsible for the spoilage of cold water marine fish.

Table 3.3 Summary of samples and isolates collected from June 2001 to March 2003 and whole cell protein extracts exposed to SDS-PAGE

	Number of samples and isolates		
	Chicken	**Meat**	**Fish**
Sampling period	2001 – 2003	2002	1996
Samples	119	37	-
Yellow-pigmented colonies	178	5	20
Strains subjected to SDS-PAGE	123	5	18

During the study, it became clear that an initial incubation of dilution plates at 4 °C for 24 hours prior to the normal incubation at 25 °C for 48 hours, retarded the strong growth of the pseudomonads and gave an opportunity for the flavobacteria to compete with the pseudomonads. Jay (2000) and Jay *et al.* (2003), however, reported that the majority of organisms do not successfully compete with *Pseudomonas* and *Acinetobacter-Moraxella* types under refrigerated conditions. This emphasises the complexicity of food on the one hand and the interaction of micro-organisms on the other. The most important factors contributing to a specific population are the storage temperature, the atmospheric composition and the fact that meat and poultry has a mildly acid pH of 5.4 to 6.4, while fish has a slightly higher pH of 6.6 to 6.8 (Forsythe, 2000).

3.3.2 SDS-PAGE protein patterns

SDS-PAGE of whole cell proteins was used to group the strains since a correlation between high DNA homology and high similarity in whole-cell protein patterns from SDS PAGE have been revealed in several studies (Vauterin *et al.*, 1993; Vandamme *et al.*, 1996) and is a recognised identification method for the flavobacteria (Bernardet *et al.*, 2002). The results of the numerical analysis of the protein profiles of 127 *Chryseobacterium/Empedobacter/Myroides* strains isolated in this study are depicted in Fig. 3.1.

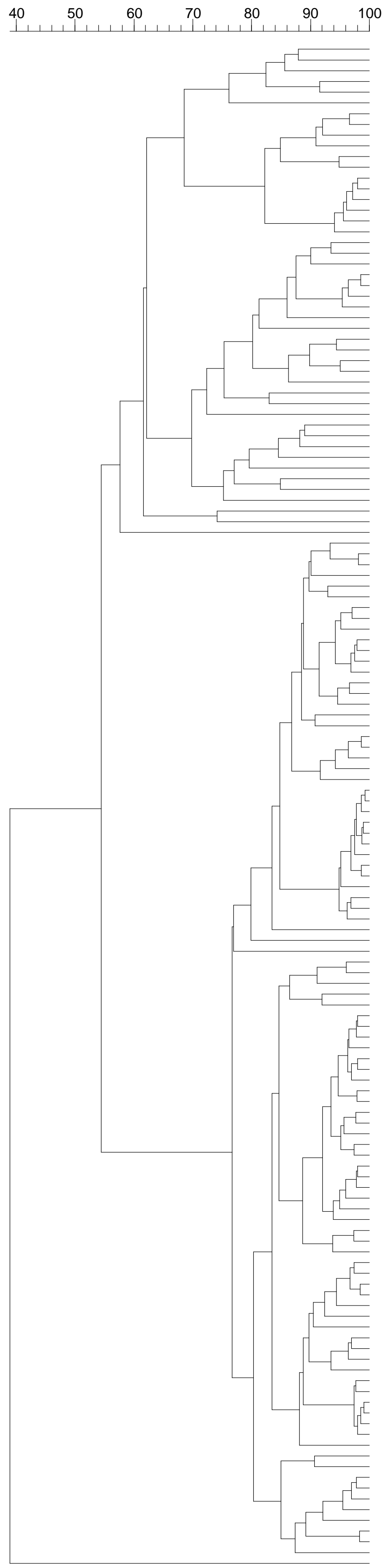
Clusters 8 and 9 representing 12 and six strains, most probably belong to the *Pseudomonaceae* group. These strains tend to be more whitish as single colonies, but yellow as a heavy inoculum on a slant culture, they tested negative for indole and only two tested positive for flexirubin pigment. They were, however, included in the study as some of the *E. meningoseptica* strains may be non-pigmented (Vandamme *et al.,* 1994). Another possibility may be that the initial *Chryseobacterium* strain was contaminated by an overlaying pseudomonad, since the single colonies were very small, and with repeated sub-culturing, the stronger strain may have dominated.

Cluster 6 consists of two strains (R-23557 and R-23535) and the *C. balustinum* reference strain. The two strains showed an association of 90 % with *C. balustinum*. These isolates were isolated from chicken.

Cluster 5 lies between the *C. balustinum* and *C. indoltheticum* reference strain clusters. It consists of four unidentifiable strains isolated from fish and will be discussed in Chapter 5 as a new species.

Cluster 7 contains four strains, isolated from chicken. It could be regarded as the *Empedobacter brevis* cluster as it has a *r*-value of 90 % with *Empedobacter brevis.*

Fig. 3.1 **Dendrogram of whole cell proteins from SDS-PAGE analysis of the isolates and reference strains based on the unweighted pair group average linkage of correlation coefficients (*r*), indicating emerging clusters.**


40 50 60 70 80 90 100
d6168 2 Chryseobac sp. R23556
d6164 4 Chryseobac sp. R23567
d6132b 15 Pseudomona sp. R23558
d6160 6 Chryseobac sp. R23485
d6137b 7 Pseudomona sp. R23490
d6132b 13 Pseudomona sp. R23555
Cluster 9
d6162 6 Chryseobac sp. R23524
d6131b 13 Pseudomona sp. R23523
d6132b 11 Pseudomona sp. R23552
d6162 8 Chryseobac sp. R23525
d6165 18 Chryseobac sp. R23521
d6137b 18 Pseudomona sp. R23522
d6137b 5 Pseudomona sp. R23487
d6136b 17 Pseudomona sp. R23561
d6137b 14 Pseudomona sp. R23498
d6133b 18 Pseudomona sp. R23599
d6160 8 Chryseobac sp. R23489
d6132b 16 Pseudomona sp. R23560
Cluster 8
d6132b 14 Chryseobac sp. R23557
d6136b 15 Chryseobac sp. R23535
Cluster 6
d6136b 8 Chryseobac balustinum LMG 8329T
d6130b 8 Chryseobac sp. R23616
d6130b 10 Chryseobac sp. R23620
d6137b 9 Chryseobac sp. R23621
d6130b 5 Chryseobac sp. R23611
Cluster 5
d6136b 2 Chryseobac indoltheticum LMG 4025T
d6133b 1 Chryseobac sp. R23605
d6162 3 Chryseobac sp. R23512
d6160 16 Chryseobac sp. R23507
d6131b 7 Empedobact sp. R23508
d6131b 8 Empedobact sp. R23513
Cluster 7
d6136b 9 Empedobact breve LMG 4011T
d6132b 4 Chryseobac sp. R23537
d6136b 7 Myroides odoratus LMG 1233T
d6136b 14 Weeksella virosa LMG 12995T
d6137b 3 Chryseobac sp. R23607
d6130b 9 Chryseobac sp. R23618
d6130b 4 Chryseobac sp. R23609
d6137b 8 Chryseobac sp. R23608
Cluster 1
d6132b 9 Chryseobac sp. R23546
d6142 4 Chryseobac miricola LMG 22470T
d6136b 13 Chryseobac meningosepticum LMG 12279T
d6130b 2 Chryseobac sp. R23606
d6137b 4 Chryseobac sp. R23612
d6137b 12 Chryseobac sp. R23625
d6162 9 Chryseobac sp. R23526
d6165 4 Chryseobac sp. R23541
d6137b 15 Chryseobac sp. R23516
d6137b 17 Chryseobac sp. R23518
d6160 4 Chryseobac sp. R23481
d6132b 6 Chryseobac sp. R23543
d6133b 16 Chryseobac sp. R23596
d6168 5 Chryseobac sp. R23590
d6160 13 Chryseobac sp. R23504
d6163 4 Chryseobac sp. R23536
d6165 5 Chryseobac sp. R23591
d6160 10 Chryseobac sp. R23495
d6165 1 Chryseobac sp. R23585
d6164 18 Chryseobac sp. R23582
d6133b 4 Chryseobac sp. R23568
d6133b 11 Chryseobac sp. R23587
d6133b 10 Chryseobac sp. R23586
Cluster 10
d6142 3 Chryseobac defluvii LMG 22469T
d6133b 9 Chryseobac sp. R23584
d6133b 14 Chryseobac sp. R23592
d6133b 15 Chryseobac sp. R23593
d6136b 5 Chryseobac scholphtalmum LMG 13028T
d6168 17 Chryseobac sp. R23597
d6132b 8 Chryseobac sp. R23545
Cluster 11
d6164 11 Chryseobac sp. R23575
d6164 13 Chryseobac sp. R23576
d6164 5 Chryseobac sp. R23569
d6164 3 Chryseobac sp. R23565
d6164 6 Chryseobac sp. R23571
d6164 8 Chryseobac sp. R23572
d6131b 4 Chryseobac sp. R23500
d6160 9 Chryseobac sp. R23493
d6160 11 Chryseobac sp. R23502
d6164 10 Chryseobac sp. R23574
d6160 5 Chryseobac sp. R23482
d6163 15 Chryseobac sp. R23563
d6164 1 Chryseobac sp. R23564
d6160 15 Chryseobac sp. R23506
Cluster 14
d6136b 12 Bergeyella zoohelcum LMG 8351T
d6131b 14 Chryseobac sp. R23527
d6163 5 Chryseobac sp. R23539
d6131b 15 Chryseobac sp. R23530
Cluster 15
d6136b 4 Chryseobac gleum LMG 8334T
d6136b 3 Chryseobac indologenes LMG 8337T
d6136b 10 Chryseobac joosteii LMG 18212T
d6168 4 Chryseobac sp. R23559
d6165 6 Chryseobac sp. R23594
d6168 3 Chryseobac sp. R23554
d6165 9 Chryseobac sp. R23598
d6165 8 Chryseobac sp. R23595
d6163 3 Chryseobac sp. R23529
d6165 10 Chryseobac sp. R23601
d6165 3 Chryseobac sp. R23588
d6165 15 Chryseobac sp. R23519
d6133b 3 Chryseobac sp. R23566
d6133b 8 Chryseobac sp. R23580
d6137b 13 Chryseobac sp. R23483
d6131b 17 Chryseobac sp. R23532
d6131b 18 Chryseobac sp. R23533
d6162 4 Chryseobac sp. R23515
d6164 15 Chryseobac sp. R23579
d6162 5 Chryseobac sp. R23517
d6160 19 Chryseobac sp. R23511
d6160 18 Chryseobac sp. R23510
d6164 19 Chryseobac sp. R23583
d6133b 13 Chryseobac sp. R23589
d6136b 18 Chryseobac sp. R23600
d6132b 19 Chryseobac sp. R23562
Cluster 4-13
d6165 13 Chryseobac sp. R23603
d6137b 2 Chryseobac sp. R23540
d6163 8 Chryseobac sp. R23542
d6163 9 Chryseobac sp. R23547
d6165 16 Chryseobac sp. R23520
d6163 10 Chryseobac sp. R23549
d6163 2 Chryseobac sp. R23528
d6132b 3 Chryseobac sp. R23534
d6132b 10 Chryseobac sp. R23551
d6165 11 Chryseobac sp. R23602
d6165 14 Chryseobac sp. R23604
d6168 12 Chryseobac sp. R23617
d6168 13 Chryseobac sp. R23619
d6168 9 Chryseobac sp. R23614
d6168 10 Chryseobac sp. R23615
d6168 7 Chryseobac sp. R23610
d6168 8 Chryseobac sp. R23613
d6168 15 Chryseobac sp. R23627
Cluster 12
d6168 14 Chryseobac sp. R23623
d6165 19 Chryseobac sp. R23553
d6164 14 Chryseobac sp. R23577
d6164 16 Chryseobac sp. R23581
d6164 9 Chryseobac sp. R23573
d6160 3 Chryseobac sp. R23480
d6160 14 Chryseobac sp. R23505
d6133b 5 Chryseobac sp. R23570
d6133b 6 Chryseobac sp. R23578
d6131b 5 Chryseobac sp. R23503
d6160 1 Chryseobac sp. R23479
Cluster 3

Cluster 1 contains four fish isolates and together with two other fish isolates with a *r*-value greater than 75 %, fall in the same cluster as *E. miricola* and *E. meningoseptica*. Additional tests on these isolates such as 16S rRNA sequencing should give better distinction between them.

Sixteen strains in Cluster 10 most probably belong to the newly described *C. defluvii* (*r* > 90 %), while the four strains in cluster 11 could be regarded as *C. scophthalmum* strains. Both Clusters 10 and 11 strains were isolated from chicken.

Cluster 15 contains two chicken isolates and clustered with the *C. gleum* reference strain at a *r*-value of 90 %.

Clusters 14, 4-13, 12 and 3 representing 13, 23, 18 and 10 strains respectively, showed high correlation with each other but could not be grouped with any reference strains and could be regarded as a possible new species or two. These clusters will be further investigated and discussed in Chapter 4 and 5. The isolates in these four clusters originated from different sources, namely chicken, fish and beef.

A few strains did not show any affiliation with any reference strain. The relation between these isolated strains and the reference strains are valued between 70 and 80 %. These values complicated a definite conclusion with regard to their position and additional tests such as 16S rRNA sequencing should indicate their genetic position and their relation to the particular reference strains. R-23546 showed a correlation of 80 % with cluster 1, R-23605 showed a correlation of 80 % with *C. indoltheticum,* while R-23537 showed a correlation of 80 % with *Myroides odoratus.* No strain showed any potential correlation with *Weeksella virosa.* R-23606 had a *r*-value of 74 % with the most closely related strains that were *E. meningoseptica* and *E. miricola*. The latter strains had a *r*-value of 80 % with each other. R-23612 and R-23625 had a *r*-value of approximately 74 % to each other and R-23526, R-23527 and R-23479 did not have a close correlation with any of the reference strains.

3.4. Conclusions

After isolation and screening of 129 Gram-negative yellow-pigmented cultures from raw meat (poultry, beef and fish), they were subjected to SDS-PAGE for grouping. It was concluded that the majority of isolates belonged to the genus *Chryseobacterium.* Eighteen flexirubin negative strains appeared to be pseudomonads while four strains belonged to *Empedobacter brevis* and only one strain to *Myroides*. Apart from three clusters that did not fall into the genus *Chryseobacterium*, 10 clusters and/or sub-clusters were members of *Chryseobacterium* of which clusters 6, 10, 11 and 15 grouped closely with *C. balustinum, C. defluvii, C. scophthalmum* and *C. gleum* respectively. Six clusters did not contain any known reference strains and could be possible new species. It can also be concluded that protein profiles on SDS-PAGE gives valuable information as a tool for classification of different micro-organisms on a genus level but further genetic information is necessary for species classification.

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CHAPTER 4

Chryseobacterium vrystaatense sp. nov., ISOLATED FROM RAW CHICKEN IN A BROILER PROCESSING PLANT

This article is accepted for publication in the International Journal for Systematic and Evolutionary Microbiology. Appendix A contains the accepted article.

Abstract

A closely related group of yellow-pigmented Gram-negative organisms, isolated from raw chicken, was investigated and after a polyphasic taxonomic approach was followed, it was evident that this group of 36 strains belongs to a new species in the genus *Chryseobacterium*. Phylogenetically, 16S rRNA sequencing of strains R-23533 and R-23566 showed a 99.9 % similarity to each other and a 97.3 %, 97.0 % and 96.9 % similarity to *C. joostei, C. indologenes* and *C. gleum* respectively. DNA-DNA hybridisations indicated that DNA binding values varied from 27 to 32 % between the test strains and the reference strains. Fatty acid methyl ester analysis and a comparative phenotypic analysis was also performed. *Chryseobacterium vrystaatense* is proposed for the new species, the G+C mol % being 37.6 % and the type strain is R-23566^T (= LMG 22846^T = CCUG 50970^T)

The EMBL accession number for the 16S rDNA sequences of *C. vrystaatense* sp. nov. (= LMG 22846^T) and LMG 22954 are AJ 871397 and AJ 871398, respectively.

4.1 Introduction

Rapid changes have taken place in the family *Flavobacteriaceae* over the past decade and several genera have been incorporated in this family, one of which being the genus *Chryseobacterium* that was proposed in the mid nineties (Vandamme *et al*., 1994). Several previously named "*Flavobacterium*" species have been included in this genus (Bernardet *et al*., 1996). Since then, several new species has been described of which *C. joostei* (Hugo *et al*., 2003) and *C. defluvii* (Kämpfer *et al*., 2003) are the newest validated members.

Flavobacteria, together with *Pseudomonas*, have been shown to cause spoilage in food and food products (Forsythe, 2000). Literature on meat spoilage, erroneously, still refers to the genus *Flavobacterium* as one of the spoilage causing bacteria as it was regarded as a generic name for yellow-pigmented rods earlier (Hendrie *et al*., 1969). Most of the food spoiling flavobacteria have, however, been grouped in the new *Chryseobacterium* genus (Bernardet *et al*., 1996). *Chryseobacterium* species are widely distributed in water, soil, the clinical environment and food commodities, such as milk, meat, poultry and fish (Jooste & Hugo, 1999).

Usually poultry are associated with pathogens like *Salmonella* and *Campylobacter,* but large numbers of other bacteria, often associated with spoilage, are found on broiler carcasses. Several Gram-negative strains are present, including *Pseudomonas* spp. and *Flavobacterium* spp. that may originate from the poultry itself or from the abattoir environment and are responsible for spoilage (Hang'ombe *et al*., 1999). Mai & Conner (2001) found that the incidence of *Pseudomonas* was 17 % and *Flavobacterium* was 16 % on broiler carcasses. The incidence of flavobacteria on poultry is much higher than on fresh red meat (Nychas & Drosinos, 1999).

In Chapter 3, a total of 128 out of 146 (approximately 88 %) yellow-pigmented colonies isolated from poultry, red meat and fish were identified as belonging to the *Flavobacteriaceae* family. After SDS-PAGE analysis, different groups of *Chryseobacterium* organisms, containing no known reference strains, emerged. These groups became possible candidates for novel *Chryseobacterium* species. The aim of this chapter will be to further investigate SDS-PAGE Clusters 4-13 and 14 by fatty acid methyl ester analysis, 16S rDNA sequence analysis, DNA-DNA hybridisations and phenotypic characterization. From the data it has become clear that a new species could be delineated and the name *Chryseobacterium vrystaatense* is proposed for this species.

4.2 Materials and Methods

4.2.1 Bacterial strains

A total of 36 strains related closely by SDS-PAGE, were isolated from raw chicken at different stages of processing, in a local Bloemfontein chicken processing plant. These strains belonged to Clusters 4-13 and 14 in Chapter 3. The strains and the reference strains used in this study are given in Table 4.1. All the strains were reactivated in nutrient broth (Oxoid CM67) and checked for purity on nutrient agar [nutrient broth + 1.5 % (w/v) agar] at 25 °C for 24 to 48 hours. The strains were maintained in a freeze-dried state on filter-paper discs and stored in screw-capped tubes at -20 °C as described previously. All isolates and reference strains were subjected to SDS-PAGE analysis according to Pot *et al.* (1994) and protein patterns were recorded in a dendrogram, with the GELCOMPAR II computer-assisted programme.

4.2.2 Fatty acid methyl ester analysis

Fatty acid methyl esters were prepared from a loopful of well-grown cells from trypticase soy agar [BBL; solidified with 1.5 % (w/v) Difco Bacto agar] after

incubation for 24 hours at 28 °C. The saponification process was done with sodium hydroxide in methanol, followed by the methylation process with hydrochloric acid in methanol. The low pH increased the volatility of the fatty acids in the column. Fatty acids were extracted in a hexane methyl t-buthyl ether solution. Separation of esters was performed in a gas chromatograph (Hewlett-Packard) with a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column, while identification of esters were done with the Sherlock Microbial Identification System (MIDI version 3.0) as previously described by Vandamme *et al.* (1992). Mean percentages and standard deviations were calculated for each taxon.

4.2.3 16S rRNA sequencing

Thirteen strains from selected SDS-PAGE groups from the previous study (Chapter 3 of this thesis) were chosen for sequencing analyses. Amplification of the 16S rRNA gene was done using conserved primers at the 5' and 3' ends of the gene and PCR products were sequenced in both directions. The sequence of the forward primer was 5'–AGAGTTTGATCCTGGCTCAG–3' and the reverse primer was 5'-AAGGAGGTGATCCAGCCGCA –3', respectively, corresponding to positions 8-27 and 1541-1522 of the *Escherichia coli* 16S rRNA numbering system. The PCR product was purified with the QIAquick PCR purification kit (Qiagen GmbH) according to the manufacturers' protocol and sequenced with a Bigdye DideoxyTerminator Cycle Sequencing kit (Perkin Elmer) and an ABI 300 Genetic Analyzer (Perkin Elmer) according to the manufacturer's instructions as described by Willems *et al.* (2003).

4.2.4 Preparation of high-molecular-weight DNA

A number of representative strains (8) from selected SDS-PAGE clusters in Chapter 3 were chosen for the DNA base composition determination and DNA-

DNA hybridisation studies. High-molecular-weight DNA was prepared according to Vandamme *et al*. (1992).

4.2.5 Determination of DNA base composition

The mean G + C values of the above mentioned organisms were determined by the thermal denaturation method (Marmur & Doty, 1962). Mol % G + C was determined with high-performance liquid chromatography using a Waters SymmetryShield C8 column thermostated at 37 °C as described by Mesbah *et al*. (1989). Calculation was done using the equation of Marmur & Doty (1962), as modified by De Ley (1970).

4.2.6 DNA-DNA hybridisations

DNA-DNA hybridisations were performed with photobiotin-labeled probes in microplate wells as described by Ezaki *et al*. (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 33 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

4.2.7 Phenotypic characterisation of isolates

Phenotypic characterisation of the isolates and *Chryseobacterium* reference species (see Table 4.1) was performed. A 24 hours culture, incubated at 25 °C, was suspended in a quarter strength Ringer solution (Oxoid BR52) to give a McFarland Barium Sulphate Standard 2 suspension. A battery of different tests was selected to differentiate between the different species in the genus at 25 °C according to the methods described by Cowan (1974), MacFaddin (1980), Gerhardt *et al.* (1981) and Hugo *et al.* (2003), as well as the Biolog GN2

MicroPlate (Biolog, Inc., Hayward, California) according to the manufacturer's protocol.

Table 4.1 Strains investigated and their origin

Strain	Origin
Proposed new species ***Chryseobacterium vrystaatense*** **sp. nov**	
R-23482	Chicken portion, 25/06/2002
R-23483	Chicken portion, 25/06/2002
R-23493	Chicken portion, 10/03/2003
R-23500	Chicken portion, 10/03/2003
R-23502	Chicken portion, 10/03/2003
R-23510	Whole bird, 17/03/2003
R-23511	Whole bird, 03/05/2003
R-23515	Whole bird, 03/05/2003
R-23517	Whole bird, 03/05/2003
R-23519	Whole bird, 03/05/2003
R-23529	Whole bird, 03/05/2003
R-23532	Manual line, 03/05/2003
R-23533	Spiral freezing process, 03/05/2003
R-23554	Automatic line, 18/02/2002
R-23559	After spin chiller, 24/02/2002
R-23562	Whole bird, 24/02/2002
R-23563	Spiral freezing process, 24/02/2002
R-23565	Spiral freezing process, 24/02/2002
R-23564	Spiral freezing process, 24/02/2002
R-23566	Portion, 24/02/2002
R-23569	Whole bird, 24/02/2002
R-23571	Whole bird, 24/02/2002
R-23572	After spin chiller, 10/02/2002
R-23574	After spin chiller, 10/02/2002
R-23575	After spin chiller, 10/02/2002
R-23576	After spin chiller, 10/02/2002

Table 4.1 continued

R-23579	Chicken portion, 10/02/2003
R-23580	Chicken portion, 10/02/2003
R-23583	Spiral freezing process, 10/02/2003
R-23588	Automatic line, 10/02/2003
R-23589	Automatic line, 10/02/2003
R-23594	Manual Cutting Process, 10/02/2003
R-23595	Manual Cutting Process, 10/02/2003
R-23598	Chicken portion, 03/03/2003
R-23600	Chicken portion, 03/03/2003
R-23601	Chicken portion, 03/03/2003
C. indoltheticum LMG 4025^T	Marine mud, 1977
C. balustinum LMG 8329^T	Fish, heart blood, 1988
C. gleum LMG 8334^T	Vaginal swab, London, 1982
C. indologenes LMG 8337^T	Human trachea at autopsy, USA, 1971
E. meningoseptica LMG 12279^T	Infant cerebral fluid, USA, 1958
C. scophthalmum LMG 13028^T	Diseased turbot gills, Scotland, 1987
C. joostei LMG 18212^T	Raw cow milk, South Africa, 1981
C. defluvii LMG 22469^T	Activated sewage sludge, Germany
E. miricola LMG 22470^T	Condensation water, space station Mir, 1997
Bergeyella zoohelcum LMG 8351^T	Human sputum, Nebraska, U.S.A, 1982
Empedobacter brevis LMG 4011^T	Human bronchial secretions, Switzerland, 1977
Flavobacterium aquatile LMG 4008^T	Deep well, Chalk region Kent, U.K., 1978
Myroides odoratus LMG 1233^T	Patent strain, 1976
Weeksella virosa LMG 12995^T	Human urine, 1992
Sphingobacterium spiritivorum LMG 8347^T	Intra-uterine specimen, 1983

LMG, BCCM/LMG Bacteria Collection Laboratory for Microbiology, University of Ghent, Belgium.

4.3 Results and Discussion

FAME and 16S rRNA results revealed that the proposed *C. vrystaatense* sp. nov. group of strains might represent a new species. It was decided, therefore, to once again subject the whole cell proteins of this group to SDS-PAGE analysis. The results of this analysis is presented in Fig. 4.1. The 36 yellow-pigmented colonies were all obtained from raw chicken, at different stages of processing, from a local broiler processing plant in Bloemfontein. The group formed three sub-clusters comprising of 19, four and 13 strains respectively.

Table 4.2 gives a summary of the predominant long chain fatty acid composition of *Chryseobacterium vrystaatense* sp. nov., in comparison with the *Chryseobacterium* reference strains. Predominant fatty acids are C15:0 iso (41.8 ±1.4), C17:1 iso ω9c (19.7±2.3) and C17:0 iso 3-OH (15.4±1.8). Summed feature 3 comprises 16:1 ω7c and/or 15:0 iso 2-OH and represents 9.1±0.9 of the total fatty acids.

4.3.1 Evidence for the proposal of a novel species

Two strains were selected to determine the 16s rRNA sequence in order to determine their position on the phylogenetic tree and their relation to closest reference neighbours. The 16S rRNA sequence of R-23533 and R-23566 (= LMG 22846^T) showed 99.9 % similarity to each other, but 97.3 %, 97.0 % and 96.9 % similarity to *C. joostei, C. indologenes* and *C. gleum* respectively.

DNA-DNA hybridisations with the two strains representing a cluster of 23 strains, and the three closest related (neighbour) reference strains, were performed. DNA binding values varied between 27 and 32 % between the test strains and the reference strains; 46 % between R-23566 and *C. joostei*; and 93 % between the two test strains (R-23533 and R-23566). A second DNA-DNA hybridisation with strain R-23566 and R-23500 (= LMG 22848) showed a DNA binding value of 92

%. R-23500 represents a cluster of 13 strains that increases the number of strains belonging to the new species to 36 strains. All these strains were subjected to conventional biochemical methods and 22 strains subjected to the Biolog GN2 MicroPlate method in order to determine their phenotypic characteristics.

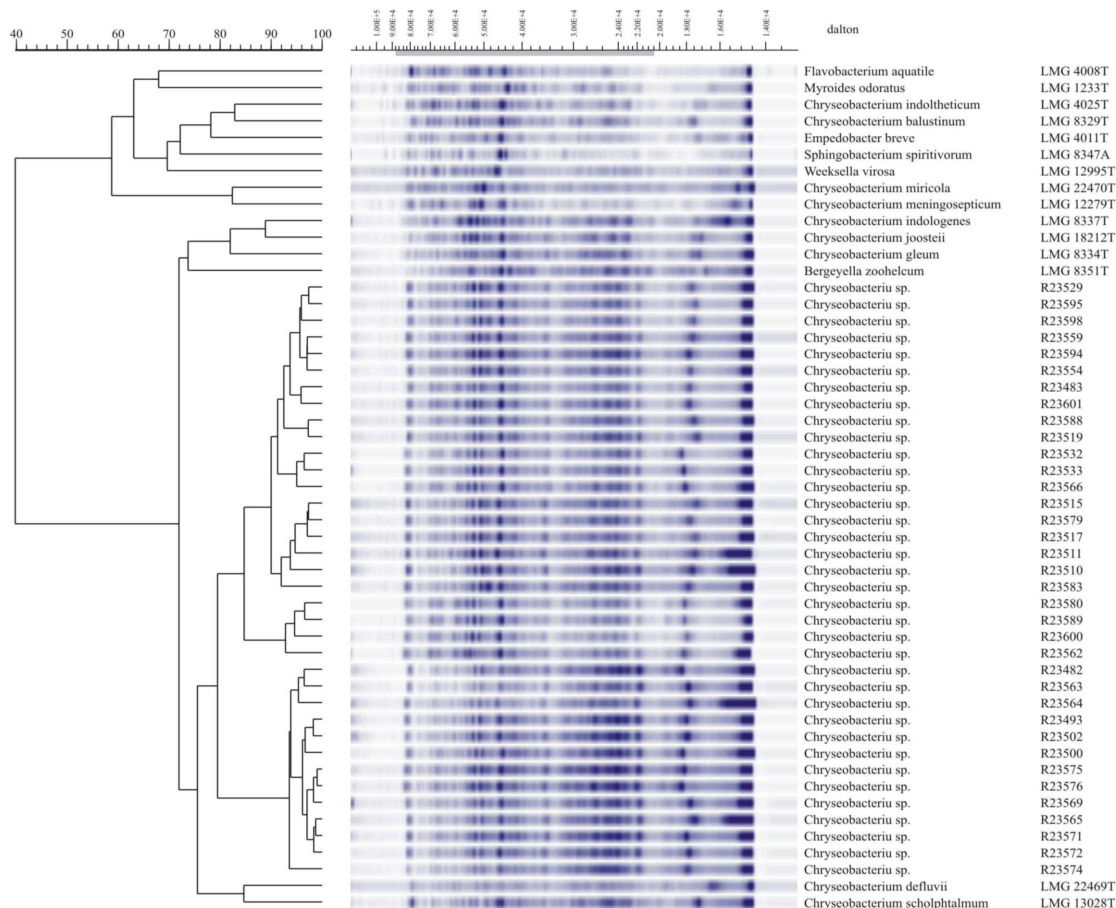

Fig. 4.1 **Dendrogram of whole cell proteins from SDS-PAGE analysis of isolates and reference strains based on unweighted pair group average linkage of correlation coefficients (*r*), indicating *C. vrystaatense.***

Table 4.2 Long chain fatty acid composition of *Chryseobacterium* species

Taxa are listed as: 1, *C. vrystaatense* sp. nov. (n=7); 2, *C. indoltheticum* (n=1); 3, *C. balustinum* (n=1); 4, *C. gleum* (n=1); 5, *C. indologenes* (n=1); 6, *E. meningoseptica* (n=1); 7, *C. scophthalmum* (n=1); 8, *C. joostei* (n=1); 9, *C. defluvii* (n=1); 10, *E. miricola* (n=1). Fatty acid percentages amounting to less than 1.0 % of the total fatty acids in all strains were not included. tr, Trace (less than 1 %); ND, not detected; ECL, equivalent chain length (i.e. the identity of the fatty acids is unknown). Means ± SD are given for *C. vrystaatense* sp. nov. only.

Fatty acid	1	2	3	4	5	6	7	8	9	10
C13:0 iso	1.1±0.4	tr	tr	tr	tr	tr	2.6	tr	tr	1.9
ECL 13.566	1.4±0.3	1.5	1.1	1.9	1.8	1.7	1.4	tr	2.5	1.3
C15:0 anteiso	1.7±0.7	tr	1.0	1.0	1.0	1.6	3.4	5.4	1.0	3.9
C15:0 iso	41.8±1.4	37.4	36.6	38.3	36.6	40.5	55.7	46.3	34.6	41.0
C15:0 iso 3-OH	2.7±0.3	2.5	2.6	2.7	2.6	2.3	2.2	3.8	4.9	2.6
C16:0	1.1±0.3	tr	tr	tr	1.3	tr	2.0	tr	1.2	tr
C16:0 3-OH	1.3±0.3	0.9	1.2	1.0	1.1	1.1	tr	tr	1.9	tr
C16:0 iso 3-OH	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
ECL 16.580	1.2±0.2	1.6	1.3	1.0	1.6	1.4	tr	tr	1.5	1.3

Table 4.2 continued

C17:0 2-OH	tr	tr	tr	tr	tr	tr	ND	tr	1.0	tr
C17:0 iso	tr	tr	tr	tr	tr	tr	3.0	tr	tr	tr
C17:0 iso 3-OH	15.4±1.8	16.7	16.0	14.5	14.8	19.5	15.5	11.3	16.9	16.8
C17:1 iso ω9c	19.7±2.3	24.1	24.7	23.6	23.5	16.9	6.4	23.3	14.3	15.5
C18:1 ω5c	tr	tr	tr	tr	tr	tr	ND	1.3	tr	1.1
Summed feature 3*	9.1±0.9	10.4	10.6	10.7	10.8	10.3	5.5	1.0	10.1	9.5
Summed feature 4*	tr	tr	tr	tr	tr	ND	ND	tr	tr	tr

*Summed feature 3 comprises 16:1 ω7c and/or 15:0 iso 2-OH.

Summed feature 4 comprises 15:0 iso 2-OH and 16: 1 ω7ct.

4.3.2 Description of *Chryseobacterium vrystaatense* sp. nov.

Chryseobacterium vrystaatense (vry.staa.ten'se, N. L. neutr. adj., named after the Vrystaat, the South African province where these bacteria were isolated).

Colonies are shiny, orange yellow and translucent as single colonies, with entire edges. Cells are Gram negative, catalase and oxidase positive and produce flexirubin with 20 % KOH, they are non-motile and neither oxidative nor fermentative. Colonies are formed after 24 hours at 4, 15, 25 and 32 °C on Nutrient agar but no growth occurs at 37 and 42 °C. Growth on MacConkey agar No. 3 (Oxoid CM115) occurs after 48 hours at 15, 25 and 32 °C for all but one strain that only grows on MacConkey agar at 32 °C after 48 hours. Growth is positive in 1 % and 2 % NaCl solution for all strains, variable reactions occur in 3 % NaCl (14 strains positive for growth). Although several strains showed growth at different salt concentrations initially, only six strains showed growth at 4 % NaCl solution after 5 days incubation (R-23493, R-23506, R-23569, R-23575, R-23576 and R-23595). Strains show strong DNase activity and also urease and lecithinase activity, but no starch and tyrosine hydrolysis. Reaction on Triple Sugar agar and 10 % lactose are alkaline. Ability to produce H_2S varies depending on time and medium. Small amounts of H_2S is produced after 3 to 5 days incubation on Triple Sugar agar by 17 strains while 23 strains produce H_2S from SIM media after 72 hours incubation and all the strains after 10 days of incubation. Table 4.3 contains a summary of the most prevalent reactions of the proposed species strains and the other reference species in the genus.

Table 4.3 Phenotypic properties that differentiate *C. vrystaatense* sp. nov. from other *Chryseobacterium* species

Taxa are listed as: 1, *C. vrystaatense* sp. nov. (n=36); 2, *C. indoltheticum* (n=1); 3, *C. balustinum* (n=1); 4, *C. gleum* (n=1); 5, *C. indologenes* (n=1); 6, *E. meningoseptica* (n=1); 7, *C. scophthalmum* (n=1); 8, *C. joostei* (n=1); 9, *C. defluvii* (n=1); 10, *E. miricola* (n=1; data from Li *et al.*, 2003). [§], Data of organism numbers 2 to 8 were from Hugo *et al.* (2003). +, Positive; ++, strongly positive reaction; w, weakly positive; -, negative; v, variable; D, delayed; NA, not available; #, number of strains tested positive.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Growth on/at:										
Cetrimide agar	+[a]	-	-	-	+	+	-	+	-	+
MacConkey agar	v[b]	+	+	+	+	+	-	+	-	+
Growth on Nutrient agar:										
5 ˚C	+	+	+	-	-	-	+	+	-	NA
15 °C	+	+	+	+	+	+	+	+	+	NA
25 °C	+	+	+	+	+	+	+	+	+	NA
32 °C	+	+	+	+	+	+	+	+	+	+
37 °C	-	-	+	+	+	+	-	-	+	-
42 °C	-	-	-	-	+	+	-	-	+	NA
3 % NaCl	14[#]	w	w	+	+	w	w	w	-	NA

Table 4.3 continued

Enzyme activity:										
DNase	++	+	+	+	+	+	+	+	+	+
Urease	+	-	-	-	-	D	+	+	-	+
Lecithinase	+	+	+	+	+	-	+	+	+	+
Nitrate reduction	-	-	+	+	-	-	-	-	-	-
Nitrite reduction	+	-	-	+	-	+	-	-	-	-
Aesculin hydrolysis	28[#]	+	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	+	+	-	-	+	w	-
Tween 80 hydrolysis	+	+	+	+	+	-	+	+	-	+
Tyrosine hydrolysis	-	D	-	+	-	D	+	+	+	-
Production of:										
Hydrogen sulphide	23[#]	+	-	-	-	-	-	-	-	+
Indole	+	+	+	+	+	D	-	-	w	+
Mean DNA G + C content (mol %) [§]	37.6	33.8	33.1	38.0	38.5	37.0	34.2	36.8	NA	34.6

Variable reactions are scored as: *a*, positive for all strains except R-23519, R-23533, R-23598; *b*, majority of strains were negative although some grew after 48 hours at 25 °C.

With the BIOLOG system, the ability of the strains to oxidize a panel of 95 different carbon sources, were tested. The following positive reactions were encountered in all the tested strains (n = 22): Tween 40 and 80, gentobiose, α-D-glucose, D-mannose, D-trehalose, succinic acid mono-methyl ester, acetic acid and L-asparagine. In the case of glycyl-L-glutamic acid, glycerol, L-glutamic acid, L-serine and L-aspartic acid, at least 19 strains (85 %) reacted positively. Reactions where more than 50 % of strains gave a positive reaction (number from 22 which tested positive indicated in brackets), are; dextrin (16), D-mannitol (18), α-ketovaleric acid (15), L-alanyl-glycine (12), L-threonine (16), inosine (13), uridine (15) and thymidine (12).

Mol % G + C for R-23566 was 37.6 % and is in the range of 34-38 mol % G + C for the *Chryseobacterium* genus (Vandamme *et al*., 1994). The type strain is R-23566 (LMG 22846^T) and was isolated from a raw chicken portion sample obtained from a broiler processing plant. The remaining 35 strains were isolated from raw chicken samples, collected at different processing stages (portion, whole bird, automatic line, manual line and the spiral freezing process; generally referred to as gyro) on different occasions.

4.4 Conclusion

After an integrated polyphasic taxonomical study, it was evident that a new species, *Chryseobacterium vrystaatense* sp.nov. had emerged.

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CHAPTER 5

Chryseobacterium piscium sp. nov., ISOLATED FROM FISH OF THE SOUTH ATLANTIC OCEAN

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Abstract

Potential *Chryseobacterium* strains have been isolated from raw meat and fish samples. After a polyphasic taxonomic study it became evident that a small group of four strains isolated from fish, belongs to a new species in the genus. Phylogenetically, 16S rRNA sequencing of strain R-23621 showed 99.3 % similarity to *C. balustinum,* 98.9 % to *C. scophthalmum* and 97.4 % to *C. indoltheticum.* DNA-DNA hybridisations with these three reference strains showed binding values of 57 %, 51 % and 52 % respectively. The name *Chryseobacterium piscium* is proposed for the new species, the G + C mol % is 33.6 % and the type strain is R-23621 (= LMG xxx; = CCUG yyy)

The EMBL accession number for the 16S rDNA sequences of *C. piscium* sp. nov. (= LMG xxx^T) is AJzzz.

5.1 Introduction

Apart from being a source of several food pathogens like *Salmonella* spp., *Vibrio* spp. and *Listeria monocytogenes*, raw fish, a protein rich food with a neutral pH (6.6 – 6.8) and high water activity, is an ideal medium for microbial growth and deterioration. Psychrotrophic, Gram-negative bacteria will dominate spoilage and members of the *Pseudomanaceae* will be the major spoilage organisms (Forsythe, 2000) although flavobacteria have also been implicated in spoilage (Chattopadhyay, 1999).

Rapid changes in the taxonomy of the family *Flavobacteriaceae* during the past decade and the description of *Chryseobacterium joostei*, isolated from milk (Hugo *et al.*, 2003), initiated an investigation into the incidence of similar yellow pigmented bacteria on raw fish. Literature still refers to *Flavobacterium* as one of the spoilage causing bacteria since it was a generic name used for Gram-negative yellow pigmented rods (Hendrie *et al*., 1969). Recently proposed *Chryseobacterium* species have proved that their habitat includes water, soil and the clinical environment and several strains have been isolated from food sources, such as milk, meat, poultry and fish (Vandamme *et al.*, 1994; Hugo *et al.*, 2003; Kämpher *et al.*, 2003; Li *et al.,* 2003; Pavlov *et al.*, 2004).

The high spoilage rate of fish can be motivated by their natural habitat, that is most often cold salt water (Forsythe, 2000). Where low temperatures are normally used to retard logarithmic growth, these conditions favour psychrotrophic organisms and optimal growth takes place, with rapid spoilage as a result. The nitrogen compounds in fish differ from the available nitrogen compounds in red meat and poultry. The ratio of protein nitrogen to the total nitrogen for fish is 0.87, implicating that non-protein nitrogen, available as free amino acids and volatile nitrogen bases such as ammonia, trimethylamine, creatine and histamine, is more susceptible to the micro-organisms and consequent deterioration (Jay, 2000). Trimethylamine is used as an indicator for the degree of fish spoilage, as it is not present in other animals (Engelbrecht *et al.*, 1996; Forsythe, 2000).

Chryseobacterium species obtained from meat, poultry and fish were subjected to SDS-PAGE analysis in Chapter 3 of this thesis, and an unidentified group of four strains, isolated from fish, was delineated from the reference strains. The aim of this chapter is to prove by further extensive research (fatty acid methyl acid analysis, 16S rRNA sequence analysis, DNA-DNA hybridisations and phenotypic characterisation), that this group is a new species. The name *Chryseobacterium piscium* is proposed for this species.

5.2 Materials and Methods

5.2.1 Bacterial strains

Potential *Chryseobacterium* strains were isolated from fresh fish from the South Atlantic ocean. These marine isolates were obtained in a previous study performed in 1996 in the Department of Food Science at the University of the Free State (Lingalo, 1997). The reference strains used were acquired from the culture collection of the University of Ghent (LMG). Table 5.1 contains a list of the reference strains used in this study as well as the four strains to be discussed in this chapter.

All the strains were maintained as freeze-dried cultures on filter paper discs (Britz & Kriel, 1973) and stored in screw capped tubes at –20 ^{o}C. Strains were reactivated in 10 ml Nutrient Broth (Oxoid CM67) and purity checked by streaking on Nutrient Agar (Oxoid CM3). Except for the initial incubation at 4 °C for 24 h, all other incubations were at 25 ^{o}C for 48 hours(Hugo & Jooste, 2003). All isolates and reference strains were subjected to SDS-PAGE analysis according to the methods as described by Pot *et al.* (1994) and protein patterns were recorded in a dendrogram, with the GELCOMPAR II computer-assisted programme as indicated in Fig 5.1.

Table 5.1 Strains investigated and their origin

Strain	Origin
***Chryseobacterium piscium* sp. nov.**	
R- 23611	South Atlantic ocean fish species, 20/02/1996
R- 23616	South Atlantic ocean fish species, 24/06/1996
R- 23620	South Atlantic ocean fish species, 24/07/1996
R- 23621	South Atlantic ocean fish species, 24/07/1996
C. indoltheticum (LMG 4025^T)	Marine mud, 1977
C. balustinum (LMG 8329^T)	Fish, heart blood, 1988
C. gleum (LMG 8334^T)	Vaginal swab, London, 1982
C. indologenes (LMG 8337^T)	Human trachea at autopsy, USA, 1971
E. meningoseptica (LMG 12279^T)	Infant cerebral fluid, USA, 1958
C. scophthalmum (LMG 13028^T)	Diseased turbot gills, Scotland, 1987
C. joostei (LMG 18212^T)	Raw milk, Kwazulu-Natal, SA, 1981
C. defluvii LMG 22469^T	Activated sewage sludge, Germany
E. miricola LMG 22470^T	Condensation water, space station Mir, 1997
Bergeyella zoohelcum LMG 8351^T	Human sputum, Nebraska, U.S.A, 1982
Empedobacter brevis LMG 4011^T	Human bronchial secretions, Switzerland, 1977
Flavobacterium aquatile LMG 4008^T	Deep well, Chalk region Kent, U.K., 1978
Myroides odoratus LMG 1233^T	Patent strain, 1976
Weeksella virosa LMG 12995^T	Human urine, 1992
Sphingobacterium spiritivorum LMG 8347^T	Intra-uterine specimen, 1983

LMG, BCCM/LMG Bacteria collection Laboratory for Microbiology, University of Ghent, Belgium.

40 50 60 70 80 90 100

d6168 2 Chryseobac sp. R23556
d6164 4 Chryseobac sp. R23567
d6132b 15 Pseudomona sp. R23558
d6160 6 Chryseobac sp. R23485
d6137b 7 Pseudomona sp. R23490
d6132b 13 Pseudomona sp. R23555
d6162 6 Chryseobac sp. R23524
d6131b 13 Pseudomona sp. R23523
d6132b 11 Pseudomona sp. R23552
d6162 8 Chryseobac sp. R23525
d6165 18 Chryseobac sp. R23521
d6137b 18 Pseudomona sp. R23522
d6137b 5 Pseudomona sp. R23487
d6136b 17 Pseudomona sp. R23561
d6137b 14 Pseudomona sp. R23498
d6133b 18 Pseudomona sp. R23599
d6160 8 Chryseobac sp. R23489
d6132b 16 Pseudomona sp. R23560
d6132b 14 Chryseobac sp. R23557
d6136b 15 Chryseobac sp. R23535
d6136b 8 Chryseobac balustinum LMG 8329T
d6130b 8 Chryseobac sp. R23616
d6130b 10 Chryseobac sp. R23620
d6137b 9 Chryseobac sp. R23621
d6130b 5 Chryseobac sp. R23611
d6136b 2 Chryseobac indoltheticum LMG 4025T
d6133b 1 Chryseobac sp. R23605
d6162 3 Chryseobac sp. R23512
d6160 16 Chryseobac sp. R23507
d6131b 7 Empedobact sp. R23508
d6131b 8 Empedobact sp. R23513
d6136b 9 Empedobact breve LMG 4011T
d6132b 4 Chryseobac sp. R23537
d6136b 7 Myroides odoratus LMG 1233T
d6136b 14 Weeksella virosa LMG 12995T
d6137b 3 Chryseobac sp. R23607
d6130b 9 Chryseobac sp. R23618
d6130b 4 Chryseobac sp. R23609
d6137b 8 Chryseobac sp. R23608
d6132b 9 Chryseobac sp. R23546
d6142 4 Chryseobac miricola LMG 22470T
d6136b 13 Chryseobac meningosepticum LMG 12279T
d6130b 2 Chryseobac sp. R23606
d6137b 4 Chryseobac sp. R23612
d6137b 12 Chryseobac sp. R23625
d6162 9 Chryseobac sp. R23526
d6165 4 Chryseobac sp. R23541
d6137b 15 Chryseobac sp. R23516
d6137b 17 Chryseobac sp. R23518
d6160 4 Chryseobac sp. R23481
d6132b 6 Chryseobac sp. R23543
d6133b 16 Chryseobac sp. R23596
d6168 5 Chryseobac sp. R23590
d6160 13 Chryseobac sp. R23504
d6163 4 Chryseobac sp. R23536
d6165 5 Chryseobac sp. R23591
d6160 10 Chryseobac sp. R23495
d6165 1 Chryseobac sp. R23585
d6164 18 Chryseobac sp. R23582
d6133b 4 Chryseobac sp. R23568
d6133b 11 Chryseobac sp. R23587
d6133b 10 Chryseobac sp. R23586
d6142 3 Chryseobac defluvii LMG 22469T
d6133b 9 Chryseobac sp. R23584
d6133b 14 Chryseobac sp. R23592
d6133b 15 Chryseobac sp. R23593
d6136b 5 Chryseobac scholphtalmum LMG 13028T
d6168 17 Chryseobac sp. R23597
d6132b 8 Chryseobac sp. R23545
d6164 11 Chryseobac sp. R23575
d6164 13 Chryseobac sp. R23576
d6164 5 Chryseobac sp. R23569
d6164 3 Chryseobac sp. R23565
d6164 6 Chryseobac sp. R23571
d6164 8 Chryseobac sp. R23572
d6131b 4 Chryseobac sp. R23500
d6160 9 Chryseobac sp. R23493
d6160 11 Chryseobac sp. R23502
d6164 10 Chryseobac sp. R23574
d6160 5 Chryseobac sp. R23482
d6163 15 Chryseobac sp. R23563
d6164 1 Chryseobac sp. R23564
d6160 15 Chryseobac sp. R23506
d6136b 12 Bergeyella zoohelcum LMG 8351T
d6131b 14 Chryseobac sp. R23527
d6163 5 Chryseobac sp. R23539
d6131b 15 Chryseobac sp. R23530
d6136b 4 Chryseobac gleum LMG 8334T
d6136b 3 Chryseobac indologenes LMG 8337T
d6136b 10 Chryseobac joosteii LMG 18212T
d6168 4 Chryseobac sp. R23559
d6165 6 Chryseobac sp. R23594
d6168 3 Chryseobac sp. R23554
d6165 9 Chryseobac sp. R23598
d6165 8 Chryseobac sp. R23595
d6163 3 Chryseobac sp. R23529
d6165 10 Chryseobac sp. R23601
d6165 3 Chryseobac sp. R23588
d6165 15 Chryseobac sp. R23519
d6133b 3 Chryseobac sp. R23566
d6133b 8 Chryseobac sp. R23580
d6137b 13 Chryseobac sp. R23483
d6131b 17 Chryseobac sp. R23532
d6131b 18 Chryseobac sp. R23533
d6162 4 Chryseobac sp. R23515
d6164 15 Chryseobac sp. R23579
d6162 5 Chryseobac sp. R23517
d6160 19 Chryseobac sp. R23511
d6160 18 Chryseobac sp. R23510
d6164 19 Chryseobac sp. R23583
d6133b 13 Chryseobac sp. R23589
d6136b 18 Chryseobac sp. R23600
d6132b 19 Chryseobac sp. R23562
d6165 13 Chryseobac sp. R23603
d6137b 2 Chryseobac sp. R23540
d6163 8 Chryseobac sp. R23542
d6163 9 Chryseobac sp. R23547
d6165 16 Chryseobac sp. R23520
d6163 10 Chryseobac sp. R23549
d6163 2 Chryseobac sp. R23528
d6132b 3 Chryseobac sp. R23534
d6132b 10 Chryseobac sp. R23551
d6165 11 Chryseobac sp. R23602
d6165 14 Chryseobac sp. R23604
d6168 12 Chryseobac sp. R23617
d6168 13 Chryseobac sp. R23619
d6168 9 Chryseobac sp. R23614
d6168 10 Chryseobac sp. R23615
d6168 7 Chryseobac sp. R23610
d6168 8 Chryseobac sp. R23613
d6168 15 Chryseobac sp. R23627
d6168 14 Chryseobac sp. R23623
d6165 19 Chryseobac sp. R23553
d6164 14 Chryseobac sp. R23577
d6164 16 Chryseobac sp. R23581
d6164 9 Chryseobac sp. R23573
d6160 3 Chryseobac sp. R23480
d6160 14 Chryseobac sp. R23505
d6133b 5 Chryseobac sp. R23570
d6133b 6 Chryseobac sp. R23578
d6131b 5 Chryseobac sp. R23503
d6160 1 Chryseobac sp. R23479

Cluster 9
Cluster 8
Cluster 6
Cluster 5
Cluster 7
Cluster 1
Cluster 10
Cluster 11
Cluster 14
Cluster 15
Cluster 4-13
Cluster 12
Cluster 3

Fig. 5.1 Dendrogram of whole cell proteins from SDS-PAGE analysis of isolates and reference strains based on unweighted pair group average linkage of correlation coefficients (*r*), indicating the proposed *C. piscium* in Cluster 5.

5.2.2 Fatty acid methyl ester analysis

Fatty acid methyl esters were prepared from a loopful of well-grown cells on Trypticase Soy agar [BBL; solidified with 1.5 % (w/v) Difco Bacto agar] and incubated for 24 hours at 28 ˚C. The saponification process was done with sodium hydroxide in methanol, followed by the methylation process with hydrochloric acid in methanol. The low pH increased the volatility of the fatty acids in the column. Fatty acids were extracted in a hexane methyl t-buthyl ether solution. Separation of esters was performed in a gas chromatograph (Hewlett-Packard) with a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column, while identification of esters were done with the Sherlock Microbial Identification System (MIDI version 3.0) as previously described by Vandamme *et al.* (1992). Mean percentages and standard deviations were calculated for each taxon.

5.2.3 16S rRNA sequencing

The phylogenetic position of this taxon was determined after amplification of the 16S rRNA gene of strain R-23621. Amplification of the 16S rRNA gene was done using conserved primers at the 5' and 3' ends of the gene and PCR products were sequenced in both directions. The sequence of the forward primer was 5'–AGAGTTTGATCCTGGCTCAG–3' and the reverse primer was 5'–AAGGAGGTGATCCAGCCGCA–3', respectively, corresponding to positions 8-27 and 1541-1522 of the *Escherichia coli* 16S rRNA numbering system. The PCR product was purified with the QIAquick PCR purification kit (Qiagen GmbH) according to the manufacturers' protocol and sequenced with a Bigdye DideoxyTerminator Cycle Sequencing kit (Perkin Elmer) and an ABI 300 Genetic Analyzer (Perkin Elmer) according to the manufacturer's instructions as described by Willems *et al.* (2003).

5.2.4 Preparation of high-molecular-weight DNA

DNA base composition determination and DNA-DNA hybridisation studies were done with strain R-23621. High-molecular-weight DNA was prepared according to Vandamme *et al.* (1992).

5.2.5 Determination of DNA base composition

The mean G + C values of the above mentioned organism was determined by the thermal denaturation method (Marmur & Doty, 1962). Mol % G + C was determined with high-performance liquid chromatography using a Waters SymmetryShield C8 column thermostated at 37 °C as described by Mesbah *et al.* (1989). Calculation was done using the equation of Marmur & Doty (1962), as modified by De Ley (1970).

5.2.6 DNA-DNA hybridisations

DNA-DNA hybridisations were performed with photobiotin-labeled probes in microplate wells as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 33 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % acetonitrille. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

5.2.7 Phenotypic characterisation of isolates

Phenotypic characterisation of the isolates and *Chryseobacterium* reference species (see Table 5.1) were performed. A 24 hours culture, incubated at 25 °C on Nutrient agar (Oxoid CM3), was suspended in a quarter strength Ringer solution to give a McFarland Barium Sulphate Standard 2 suspension. A battery of different tests was selected to differentiate between the different species in the genus at 25 °C according to the methods described by Cowan (1974), MacFaddin (1980), Gerhardt *et al.* (1981) and Hugo *et al.* (2003), as well as the Biolog GN2

MicroPlate (Biolog, Inc., Hayward, California) according to the manufacturer's protocol.

5.3 Results and Discussion

Table 5.2 gives a summary of the predominant long chain fatty acid composition of the proposed *Chryseobacterium piscium* sp. nov., in comparison with the other *Chryseobacterium* reference strains. The average proportion of the predominant fatty acids were C15:0 iso (38.3 %), C17:1 iso ω9c (18.7 %), C17:0 iso 3-OH (16.2 %) and summed feature 3 (10.8 %).

5.3.1 Evidence for the proposal of a novel species

The 16S rRNA sequence of strain R-23621 was determined and the results with regard to the position on the phylogenetic tree and the relation with the closest reference neighbours was recorded in a dendrogram. The 16S rRNA sequence of R-23621 showed 99.3 % similarity to *C. balustinum,* 98.9 % to *C. scophthalmum* and 97.4 % to *C. indoltheticum*. DNA-DNA hybridisations with these three reference strains showed binding values of 57 %, 51 % and 52 % respectively. R-23621 represents a small cluster that also includes strains R-23611, R-23616 and R-23620. These four strains have been subjected to testing by conventional biochemical methods and the Biolog GN2 MicroPlate method in order to determine their phenotypic characteristics.

Table 5.2 Long chain fatty acid composition of *Chryseobacterium* species

Taxa are listed as: 1, proposed *C. piscium* sp. nov. (n=4); 2, *C. indoltheticum* (n=1); 3, *C. balustinum* (n=1); 4, *C. gleum* (n=1); 5, *C. indologenes* (n=1); 6, *E. meningoseptica* (n=1); 7, *C. scophthalmum* (n=1); 8, *C. joostei* (n=1); 9, *C. defluvii* (n=1); 10, *E. miricola* (n=1). Fatty acid percentages amounting to less than 1.0 % of the total fatty acids in all strains were not included. Trace (tr) for values less than 1 %; ND, not detected; ECL, equivalent chain length (i.e. the identity of the fatty acids was unknown). Means ± SD are given for proposed *C. piscium* sp. nov. only.

Fatty acid	1	2	3	4	5	6	7	8	9	10
C13:0 iso	0.9±0.6	tr	tr	tr	tr	tr	2.6	tr	tr	1.9
ECL 13.566	tr	1.5	1.1	1.9	1.8	1.7	1.4	tr	2.5	1.3
C15:0 anteiso	2.7±1.9	tr	1.0	1.0	1.0	1.6	3.4	5.4	1.0	3.9
C15:0 iso	38.3±5.0	37.4	36.6	38.3	36.6	40.5	55.7	46.3	34.6	41.0
C15:0 iso 3-OH	2.4±0.3	2.5	2.6	2.7	2.6	2.3	2.2	3.8	4.9	2.6
C16:0	1.1±0.2	tr	tr	tr	1.3	tr	2.0	tr	1.2	tr
C16:0 3-OH	1.3±0.3	0.9	1.2	1.0	1.1	1.1	0.5	0.6	1.9	0.8
C16:0 iso 3-OH	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
ECL 16.580	1.2±0.2	1.6	1.3	1.0	1.6	1.4	tr	tr	1.5	1.3

Table 5.2 continued

C17:0 2-OH	1.2±1.2	tr	tr	tr	tr	tr	ND	tr	1.0	tr
C17:0 iso	tr	tr	tr	tr	tr	tr	3.0	tr	tr	tr
C17:0 iso 3-OH	16.2±3.1	16.7	16.0	14.5	14.8	19.5	15.5	11.3	16.9	16.8
C17:1 iso ω9c	18.7±2.8	24.1	24.7	23.6	23.5	16.9	6.4	23.3	14.3	15.5
C18:1 ω5c	tr	tr	tr	tr	tr	tr	ND	1.3	tr	1.1
Summed feature 3#	10.8±1.3	10.4	10.6	10.7	10.8	10.3	5.5	1.0	10.1	9.5
Summed feature 4#	tr	tr	tr	tr	tr	ND	ND	tr	tr	tr

Summed feature 3 comprises of 16:1 ω7c and/or 15:0 iso 2-OH.

Summed feature 4 comprises of 15:0 iso 2-OH and 16: 1 ω7ct.

5.3.2 Description of *Chryseobacterium piscium* sp. nov.

Chryseobacterium piscium (L. pl. gen. n. of fish)

Colonies are shiny, yellow and translucent as single colonies, with entire edges. Cells are Gram negative, catalase and oxidase positive and produce flexirubin type pigment with 20 % KOH., They are non-motile and neither oxidative nor fermentative. Colonies are formed after 24 hours at 4, 15, 25 °C. At 32 °C, very weak growth occurs on Nutrient agar, but no growth occurs at 37 and 42 °C. No growth occurs on MacConkey No. 3 (Oxoid CM115) agar. Strains have the ability to grow in a 5 % NaCl solution in nutrient broth. Strong production of aesculin occur within 24 h. Strains test positive for phenyl alanine deaminase activity, gelatine hydrolysis, casein hydrolysis, lecithinase and phosphatase activity and produce urea from ammonia. Weak DNase activity occur but no starch or tyrosine hydrolysis occur. Reaction on triple sugar agar (Oxoid CM277) and 10 % lactose were alkaline and strains did not produce H_2S from triple sugar agar or SIM medium (Oxoid CM435). Table 5.3 contains a summary of the differentiating characteristics of the proposed species and the other reference species in the genus.

With the BIOLOG system the ability of the strains to oxidize a panel of 95 different carbon sources, was tested. Only four positive reactions were encountered in all the tested strains (n = 4), namely gentobiose, D-mannose, succinic acid mono-methyl ester and acetic acid.

Mol % G + C for R-23621 was 33.6 % and is in the range of 34-38 mol % G + C for the *Chyseobacterium* genus (Vandamme *et al.*, 1994).

The type strain is R-23621 (= LMG XXX), and together with the other three strains, was isolated from fish from the South Atlantic ocean off the South African coastline during 1996.

Table 5.3 Phenotypic properties that differentiate *C. piscium* sp. nov. from other *Chryseobacterium* species

Taxa are listed as: 1, proposed *C. piscium* sp. nov. (n = 4); 2, *C. indoltheticum* (n = 1); 3, *C. balustinum* (n = 1); 4, *C. gleum* (n = 1); 5, *C. indologenes* (n = 1); 6, *E. meningoseptica* (n = 1); 7, *C. scophthalmum* (n = 1); 8, *C. joostei* (n = 1); 9, *C. defluvii* (n = 1); 10, *E. miricola* (n = 1; data from Li *et al.*, 2003); 11, *C.vrystaatense* sp. nov. (n = 36). +, Positive; ++, strong positive reaction; w, weakly positive; -, negative; v, variable; D, delayed; NA, not available; #, number of strains tested positive.

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Growth on:											
Cetrimide agar	-	-	-	-	+	+	-	+	-	+	33#
MacConkey agar	-	+	+	+	+	+	-	+	-	+	v
Growth on Nutrient agar at:											
5 ˚C	+	+	+	-	-	-	+	+	-	NA	+
15 °C	+	+	+	+	+	+	+	+	+	NA	+
25 °C	+	+	+	+	+	+	+	+	+	NA	+
32 °C	w	+	+	+	+	+	+	+	+	+	+
37 °C	-	+	+	+	+	+	-	-	+	-	-
42 °C	-	-	-	-	-	+	-	-	-	NA	-
5 % NaCl	+	-	-	-	-	-	-	-	-	NA	-

Table 5.3 continued

Enzyme activity:											
DNase	w	+	+	+	+	+	+	+	+	+	++
Urease	+	-	-	-	-	D	+	+	-	+	+
Lecithinase	+	+	+	+	+	-	+	+	+	+	+
Nitrate reduction	+	-	+	+	-	-	-	-	-	-	-
Nitrite reduction	-	-	-	+	-	+	-	-	-	-	+
Aesculin hydrolysis	++	+	+	+	+	+	+	+	+	+	28#
Starch hydrolysis	-	-	-	+	+	-	-	+	w	-	-
Tween 80 hydrolysis	v	+	+	+	+	-	+	+	-	+	+
Tyrosine hydrolysis	-	D	-	+	-	D	+	+	+	-	-
Phenyl alanine deaminase	+	+	-	-	-	+	-	-	-	NA	-
Production of:											
Hydrogen sulphide	-	+	-	-	-	-	-	-	-	+	23#
Indole	+	+	+	+	+	D	-	-	w	+	+
Mean DNA G+ C content (mol %)##	33.6	33.8	33.1	38.0	38.5	37.0	34.2	36.8	34.6	NA	37.6*

Values from Hugo *et al.*, 2003 (2-8).

5.4 Conclusion

After an integrated polyphasic taxonomical study, it became evident that the four strains R-23611, R-23616, R-23620 and R-23621 belong to a new species, and the name *Chryseobacterium piscium* sp.nov. is proposed.

5.5 Acknowledgments

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CHAPTER 6

POTENTIAL SOURCES OF *Chryseobacterium* CONTAMINATION DURING POULTRY PROCESSING

6.1 Introduction

Specific micro-organisms are associated with food spoilage and resultant metabolic products such as slime production, ammonia and sulphur compounds responsible for undesirable flavours and odours (Sundheim *et al.,* 1998; Gram *et al.*, 2002). Competition between the different micro-organisms on the food, as well as the temperature of storage, will also have an effect on the population and the expected deterioration (Forsythe, 2000; Jay, 2000). Despite these organisms' source of origin, which may be from the poultry itself or from the abattoir environment, they can be responsible for spoilage (Hang'ombe *et al.*, 1999) with a resultant economic as well as a possible health impact on both industry and consumers.

Micro-organisms enter the poultry processing plant through feathers, skins and intestines and accumulate in processing water (Mead, 1989). Several processing steps reduce the contamination levels but equipment and operating procedures contribute to the microbiological load of the poultry end product (Geornaras *et al.*, 1996). A significant increase of psychrotrophic spoilage bacteria during processing is due to cross-contamination from the air, water, handling practices and intestines. These bacteria are responsible for spoilage of poultry during refrigerated storage (Hinton *et al.,* 2004).

Psychrotrophic bacteria have the ability to grow at 5 ^{o}C and they can be responsible for food spoilage in food stored under refrigerated temperatures. Several studies revealed that apart from the Gram-positive strains, several Gram-negative strains are present on poultry, including *Pseudomonas* spp., *Flavobacterium* spp., *Alcaligenes* spp., *Klebsiella* spp. and *E. coli* (Geornaras *et al.*, 1996; García-López *et al.*, 1998, 1999; Hang'ombe *et al.*, 1999; Hinton *et al.*, 2004; Vazgecer *et al.*, 2004). Pseudomonads as well as the flavobacteria, are

Gram-negative, aerobic rods capable of growing well under refrigerated conditions. Mai & Conner (2001) found that the incidence of *Pseudomonas* and *Flavobacterium* on chicken carcasses were 17 % and 16 % respectively. Nychas & Drosinos (1999), found that the incidence of flavobacteria on poultry is much higher than on fresh meat.

During the past decade, the family *Flavobacteriaceae* has emerged to accommodate the Gram-negative yellow rods and since its validation in 1992, the genus *Flavobacterium* has become only one of several genera in this family. The genus *Chryseobacterium* is widely distributed in the environment and is often associated with food spoilage (Bernardet *et al.*, 1996). Due to this fairly new reclassification, literature with regard to food spoilage, erroneously persists in referring to *Flavobacterium*/flavobacteria as psychrotrophic bacteria responsible for spoilage.

In Chapter 3 of this thesis, several *Chryseobacterium* species were isolated from chicken carcasses from different processing areas in a chicken abattoir. The aim of this part of the study was, therefore, to investigate the possible source(s) of *Chryseobacterium* contamination in the poultry processing plant.

6.2 Materials and Methods

6.2.1 Sampling points

A Grade A poultry abattoir with a slaughtering capacity of 65 000 to 67 000 birds per day, was sampled in this study (Blignaut, 2001). Birds slaughtered came from contracted poultry farmers or the company's own farm. Microbiological contamination of the air, bird carcasses and water used during processing was investigated. A simplified flow diagram of the processing steps and sampling points in the abattoir under investigation is indicated in Fig. 6.1. All samples were chosen at random from the production line at 5 min. intervals between the different sampling points.

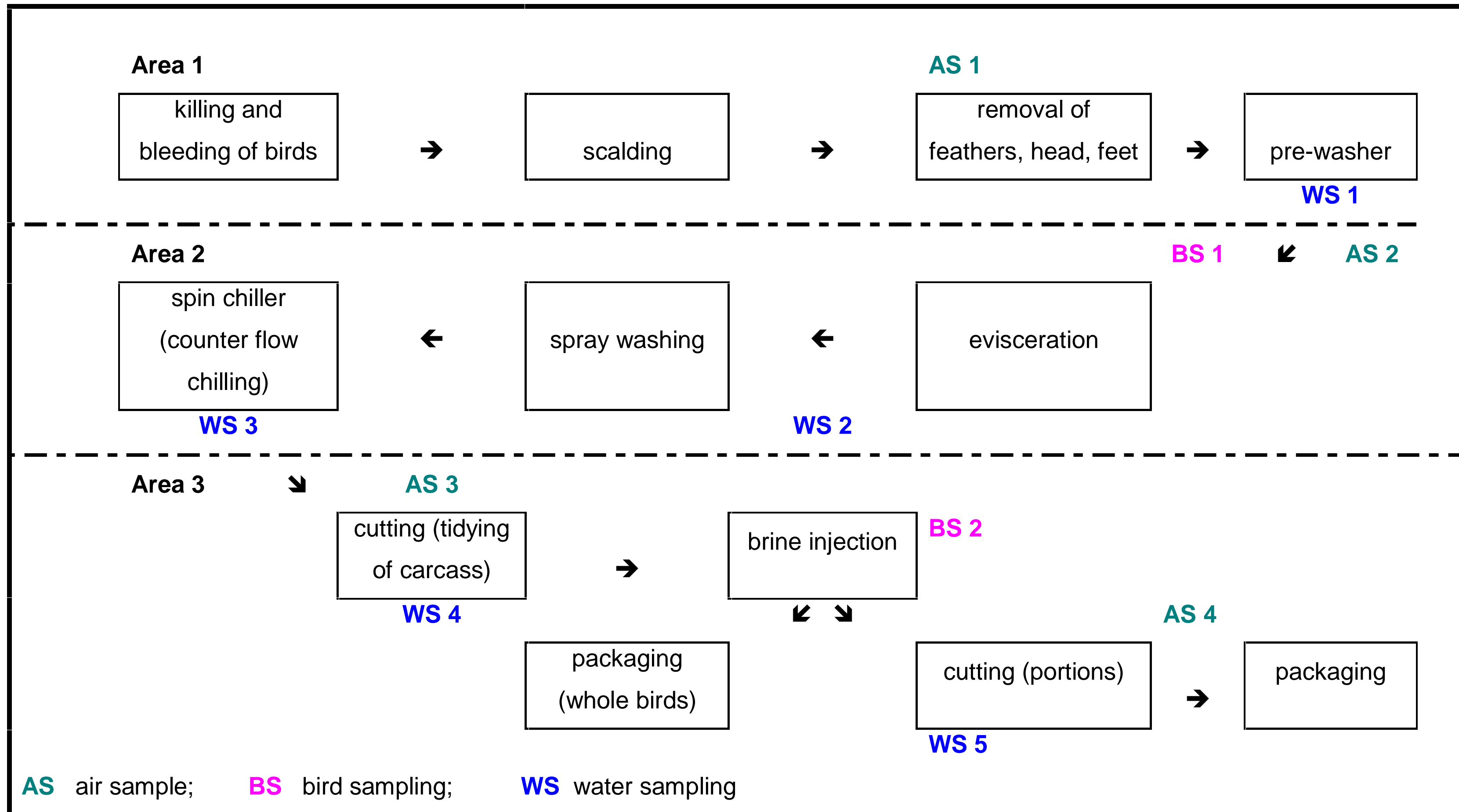

Fig. 6.1 Poultry processing flow diagram and sampling points.

6.2.2 Sample collection

6.2.2.1 Surface swab sampling

Surface swab samples on whole bird carcasses were taken at two different stages in the production line, namely after the first washing step but before evisceration and then after the brine injection stage. Each carcass was swabbed in the same area, namely the neck, the back, the right wing and the right thigh. Five birds were sampled at bird sampling point 1 and three birds were sampled in the same way at the second bird sampling point. Three samples from the internal part of each carcass were also taken. Swabs were immersed in 9 ml sterile quarter strength Ringer solution (Oxoid BR52). Table 6.1 contains a summary of the sampling plan.

6.2.2.2 Processing water

Water samples were taken at different stages of the production process, namely; before any processing, after evisceration, beginning of the pre-washer, end of the spin chiller and several areas where cutting of the carcasses for portions took place (Table 6.1).

6.2.3 Microbial analysis

6.2.3.1 Total bacteria and yellow-pigmented colony counts

Serial dilutions were prepared in 9 ml quarter strength Ringers' solution (Oxoid BR52) and spread-plated onto Plate count agar (Oxoid CM463). All the plates were incubated at 4 °C for 48 hours and then at 25 °C for 48 hours as explained in Chapter 3 of this thesis. Total plate counts, as well as yellow colony counts were recorded from plates with counts of between 30 and 300 colonies.

Table 6.1 Summary of the different samples taken at each sampling point

Sampling point	Number of samples	Comments
Surface swab (1cm^2)	**n = 41**	
BS 1	20	Five birds, external swabs (four areas on carcass)
BS 2	21	Three birds, external & internal (four areas on carcass, three inside)
Water sample	**n = 31**	
WS 1	7	Three before contact with carcass; four during process – water dripping from carcass
WS 2	4	Two before contact with carcass; two during evisceration – water dripping from carcass
WS 3	4	Two at start of process; two at end of spin chilling process
WS 4	8	Four before contact with carcass; four during cutting process – water dripping from carcass
WS 5	8	Four before contact; four after portions cut – water dripping from portion
Air sample	**n = 4**	
AS 1	1	Prepared PCA, 20 min exposure to air in that section
AS 2	1	Prepared PCA, 20 min exposure to air in that section
AS 3	1	Prepared PCA, 20 min exposure to air in that section
AS 4	1	Prepared PCA, 20 min exposure to air in that section

PCA; Plate count agar

6.2.3.2 Screening for Chryseobacterium species

All yellow and orange-yellow colonies were inoculated on Brilliant Green Agar (Oxoid CM263) and incubated at 25 °C for 48 hours. Yellow colonies turning the surrounding medium red were regarded as potential *Chryseobacterium* species.

Confirmation of *Chryseobacterium* species, were done by using the screening tests as described in Chapter 3 of this thesis.

6.2.4 Statistical analysis

Statistical analyses were done on actual counts with the multiple comparison analysis (F-test) and a confidence interval of 95 % using Excel statistics. A two tail comparison was used as it is commonly used for biological data.

6.3 Results and Discussion

The main aim of this investigation was to determine whether *Chryseobacterium* species are introduced to poultry through environmental sources or from the poultry itself and therefore the focus was on the carcasses and the processing water. Four air samples were obtained only to establish what the situation in the processing area was. Mead (1989) and Geornaras *et al.* (1996) reported on the role of cross-contamination by psychrotrophic bacteria during processing from equipment, processing water and handling. The occurrence of *Flavobacterium* and *Cytophaga* species during poultry processing was reported. Both of the latter organisms are genera in the “newly” described family *Flavobacteriaceae*, as discussed in detail in Chapter 2 of this thesis, and it is necessary to establish the prevalence of *Chryseobacterium* on raw meat before one would investigate their specific role in food spoilage.

Table 6.2 contains a summary of the colony counts as they were observed during the study. Counts for control samples were not included in the statistical analysis. It was clear that no statistically significant differences were found between the total organisms found on different areas on the carcasses, but variation in numbers at different processing areas were significant.

Table 6.2 Summary of Total Plate Counts, total yellow colonies and total *Chryseobacterium* colonies obtained from surface swab samples in the chicken processing area

		Average Total Counts **(cfu/ml or cfu/cm²)**		**Average Percentage (%)**	
Sample	**n**	**Plate Count**	***Chryseobacterium* Counts**	**Yellow colonies**	***Chryseobacterium***
Surface swabs from birds (cfu/cm²)					
After scalding[a]	**20**	9 250	515	11.2	6.2
Std dev.		7 800	500		
Multiple comparison analysis between 5 birds (counts on each bird): $F_{test} = 0.25 < F_{crit}$ (3.81)					
after brine injection[b]	**12**	3 541	417	18.2	12.0
Std dev.		3 100	400		
Multiple comparison analysis between 3 birds (counts on each bird): $F_{test} = 0.66 < F_{crit}$ (5.71)					
after brine; intestines[c]	**9**	9 110	417	7.0	4.6
Std dev.		5 200	400		
Multiple comparison analysis between 3 birds (internal counts): $F_{test} = 2.57 < F_{crit}$ (4.77)					
after brine; total[d]	***21***	*6 326*	*417*	*12.6*	*8.3*

Two tail comparison between a and b for Total counts: $F_{test} = 6.35 > F_{crit}$ (2.66)

Two tail comparison between a and b for Total *Chryseobacterium* counts: $F_{test} = 0.56 > F_{crit}$ (0.42)

Two tail comparison between b and c for Total counts: $F_{test} = 0.35 > F_{crit}$ (0.34)

Two tail comparison between b and c for Total *Chryseobacterium* counts: $F_{test} = 0.85 > F_{crit}$ (0.34)

Washing processes after the scalding process reduced the microbial contamination significantly ($p < 0.05$ for the total counts on birds and total *Chryseobacterium* counts), even if one included the intestinal surface counts. It was actually expected to contribute to higher total counts of whole birds. This corresponded with a study done by Hinton *et al.* (2004) who determined the extent and types of spoilage bacteria on processed broiler carcasses, although they used different agar media. In a study on fish skin samples it was found that during plate cultivation, *Flavobacterium* species were masked by other saprophytic species and that pseudomonads could inhibit the growth of flavobacteria (Tiirola *et al.*, 2004).

Table 6.3 contains information on the bacterial load of the processing water. Fresh potable water was introduced at several processing areas throughout the plant. Samples were collected at different processing areas and at the different sampling areas. The increase in bacterial numbers was significant ($p < 0.05$).

During processing of raw chicken the load of micro-organisms in the processing water increased and specifically during the washing processes after evisceration although the incidence of *Chryseobacterium* was only 2 %. As water is frequently replaced with new potable water, contamination levels fluctuated in different processing areas. At two stages, where cutting of the carcasses were involved (after the evisceration process and the portioning line) a significant increase in the total bacterial count, as well as total *Chryseobacterium* count occurred. The average total counts at these two processes were 2.5×10^3 cfu/ml for total bacterial counts and 3.4×10^4 cfu/ml with *Chryseobacterium* species representing 20.4 and 25.2 % of the population. Further investigation will be needed to determine if the higher numbers are caused by growth during processing or whether the bird itself, for example via the bird's internal fluids, may be responsible for this contamination. It seemed, however, more likely that the birds contribute to these higher counts rather than growth, as running tap water was used during the cutting process.

Table 6.3 Summary of Total Plate Counts, total yellow colonies and total *Chryseobacterium* counts obtained from water samples in the chicken processing area

		Average Total Counts (cfu/ml or cfu/cm^2)		Average Percentage (%) of Total Bacterial Count	
Sample	**n**	**Plate Count**	***Chryseobacterium* Count**	**Total Yellow colonies**	***Chryseobacterium***
Water cfu/ml					
before processing	**11**	10	0	1.0	0
Std dev.		*0*	*0*		
after evisceration	**5**	42 800	1 800	4.2	2.0
Std dev.		*20 000*	*800*		
prewasher start	**4**	252 500	4 251	3.5	1.7
Std dev.		*50 000*	*4 000*		
spin chiller end	**4**	80	0	1.3	0
Std dev.		*10*	*0*		
after cutting	**4**	2 540	500	20.4	20.4
Std dev.		*2 000*	*450*		
after portions	**4**	33 725	9 500	31.8	25.2
Std. dev.		*3 000*	*4 000*		

F-test for two-sample variance

Before processing: after processing – Total counts $F_{test} = 21.24 > F_{crit}$ (3.73)

Before processing: after processing – Total *Chryseobacterium* counts $F_{test} = 0.48 > F_{crit}$ (0.29)

In a study in Germany on the incidence of airborne microflora during poultry slaughtering, several Gram-positive strains were isolated in the area of the evisceration machine. It was found that *Flavobacterium* spp., representing the Gram-negative bacteria, predominated in the reception area, while *Staphylococcus* and *Micrococcus* species represented the Gram-positive organisms (Ellerbroek, 1997). In this study, the focus was on *Chryseobacterium* species and air samples indicated a relative low incidence, but definite presence of *Chryseobacterium* species in all areas of processing. High loads of bacteria are carried in the air and this can be seen in contamination levels on agar plates in the different processing areas. The agar plate representing the air sample in the bird reception area was overgrown, but air samples from the other three areas indicated much lower contamination levels. The two air samples obtained from area 3 (Fig. 6.1) actually had low total counts, which was an indication of good hygiene management during the final processing stage.

A conclusion that can be reached is that the outside environment via dust on feathers, is a source of contamination of *Chryseobacterium* and other bacteria.

6.4 Conclusion

During this study it was evident that *Chryseobacterium* species were present throughout the processing unit of a poultry processing plant. Environmental sources such as dust, most likely contributed to contamination levels of psychrotrophic, yellow-pigmented colonies and especially *Chryseobacterium*, on raw chicken meat. Even though the washing processes lowered total contamination by these spoilage organisms, *Chryseobacterium* species contributed to 8.3 % of the total bacterial count on whole birds in this study. It was also found that a significant increase in total counts, as well as total *Chryseobacterium* counts (25.2 %) in processing waters occurred during processes where cutting of the carcasses was involved. It is, therefore, recommended that live chickens, as a source of *Chryseobacterium* contamination, will be investigated in future studies. This part of the study also emphasizes the importance of microbial diversity and the interaction of psychrotrophic bacteria on different raw food products during storage. Future research needs to investigate

the role of *Chryseobacterium* species as spoilage organism, as well as their interaction with other organisms, especially pseudomonads during refrigerated storage.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The taxonomy of the flavobacteria has undergone many changes since its inception in 1923 (Bernardet *et al.*, 1996). During the past decade, *Flavobacteriaceae* has emerged as a family for a variety of Gram-negative yellow-pigmented rods and *Flavobacterium* has become only one of several genera in this family (Bernardet *et al.*, 2002). Due to this fairly new reclassification, literature with regard to food spoilage still refers to psychrotrophic bacteria of this group involved in spoilage as *Flavobacterium* or flavobacteria or CDC Group IIb organisms. The present situation, however, is that genera in the *Flavobacteriaceae* family associated with food spoilage should more correctly be designated *Bergeyella*, *Chryseobacterium, Empedobacter, Myroides* or *Weeksella*, (Hugo & Jooste, 2003).

During this study, a polyphasic taxonomical approach as described by Vandamme *et al.* (1996) was followed. This approach integrates phenotypic and genotypic testing methods and makes it possible to classify strains into the existing taxonomical structures with greater precision.

7.1 Food spoilage

Microbial diversity in a food commodity complicates the prediction or measurement of the effect of specific micro-organisms on specific food types stored under different conditions. According to Gram & Dalgaard (2002), the field of food microbiology was static for several years since the industry was satisfied with total counts and absence of certain food pathogens as sufficient criteria for quality. Introduction of molecular techniques led to a new dimension of refining bacterial taxonomy and to the integration of molecular, microbiological, biochemical and sensory analysis of food. This provides added scope for exploring the complex research field of the microbiological ecology of foods.

Giraffa & Neviani (2001) claimed that food is an unexplored source of bacterial diversity and that several organisms isolated from food have been incorrectly classified. Apart from the fact that the classification of many Gram-negative organisms has changed, the diversity referred to in food still remains relevant. This limits the appropriate literature on members of the family *Flavobacteriaceae* and specifically *Chryseobacterium* that have been isolated from food sources. When the genus *Chryseobacterium* was proposed by Vandamme *et al.* (1994), former *Flavobacterium* species renamed as *Chryseobacterium* were *Chryseobacterium* [*Flav.*] *indologenes, Chryseobacterium* [*Flav.*] *gleum, Chryseobacterium* [*Flav.*] *indoltheticum*, *Chryseobacterium* [*Flav.*] *balustinum, Chryseobacterium* [*Flav.*] *meningosepticum.* During 2003 new species proposed and validated were *C. joostei* (Hugo *et al.*, 2003), *C. defluvii,* (Kämpfer *et al.*, 2003) and *C. miricola,* (Li *et al.*, 2003). *Chryseobacterium joostei* was the first Chryseobacterium species isolated from a food commodity. Other currently validated *Chryseobacterium* species that are often associated with food are *C. balustinum, C. gleum* and *C. indologenes* (Hugo & Jooste, 2003). The three most recently published new species; *C. formosense* (Young *et al.*, 2005), *C. daecheongense* (Kim *et al.*, 2005) and *C. taichungense* (Shen *et al.*, 2005) were not isolated from food.

7.2 Isolation of Gram-negative yellow-pigmented colonies

After isolation of several Gram-negative yellow-pigmented colonies of the family *Flavobacteriaceae* from red meat, poultry and fish, results from SDS-PAGE analysis in Chapter 3 of this thesis indicated that the majority of strains were members of the genus *Chryseobacterium*. During the isolation stage of this research it was observed that the number of yellow colonies on agar plates varied considerably. After pure cultures of each isolate were obtained, it turned out that a large proportion of isolates were Gram-positive. Introduction of Brilliant Green agar eliminated the Gram-positive strains. Initial incubation at 4 °C for 48 hours gave increased selection of the more psychrophilic organisms. After subjection to a screening regime for potential *Flavobacteriaceae* isolates*,* a total of 129 Gram-negative yellow-pigmented pure cultures were isolated from raw poultry, red meat and fish samples.

Protein fingerprint profiles resulting from SDS-PAGE analysis indicated that the majority of the isolates belonged to the genus *Chryseobacterium.* Eighteen flexirubin-negative strains appeared to be pseudomonads, while 4 strains belonged to *Empedobacter brevis* and only 1 strain to *Myroides*. Apart from the 3 clusters that did not fall into the genus *Chryseobacterium*, 10 clusters and/or sub-clusters were members of the genus *Chryseobacterium.* Several strains isolated from the meat sources could be equated with *C. balustinum* (2)*, C. defluvii* (16), *C. scophthalmum* (4) or *C. gleum* (2) reference strains. Nine strains did not show affiliation with any reference strain. The relationship between the clustered strains and the relevant reference strain, ranged between 70 and 80%. These values were unable to allow definite conclusions with regard to their identities and additional tests such as 16S rRNA sequencing were required to indicate their genetic position and their exact relationship to the particular reference strains. The strains implicated were R-23546 which showed a correlation of 80% with cluster 1; R-23605 which showed a correlation of 80% with *C. indoltheticum*; R-23537 showed a correlation of 80% with *Myroides odoratus*; R-23606 which had a *r*-value of 74% with the most closely related strains (*E. meningoseptica* and *C. miricola)*; R-23612 and R-23625 had a *r*-value of approximately 74% with each other and R-23526, R-23527 and R-23479 which did not have a close correlation with any of the reference strains.

7.3 ‘*Chryseobacterium vrystaatense* sp.nov.’

After an integrated polyphasic taxonomical study, it became evident that a proposed new species named *Chryseobacterium vrystaatense* sp.nov. had emerged. The characterization of this species is discussed in Chapter 4. The type strain of this new species, namely R-23566 (= LMG 22846^{T}), as well as the other 35 strains of this species were isolated from raw chicken samples, collected at different processing stages (portioning, whole bird, automatic line, manual line and the spiral freezing process; generally referred to as gyro) on different occasions. Phylogenetically, 16S rRNA sequencing of strains showed R-23533 and R23566 to have a 99.9 % similarity with each other and a 97.3 %, 97.0 % and 96.9 % similarity with *C. joostei, C. indologenes* and *C. gleum* respectively. DNA-DNA hybridisations indicated that DNA binding values varied from 27 to 32 %

between the test strains and the reference strains in this specific group. Mol % G + C for R-23566 was 37.6 % and is in the range of 34-38 mol% for the *Chyseobacterium* genus (Vandamme *et al.*, 1994). Only five of the 36 strains (R-23493, R-23569, R-23575, R-23576 and R-23595) showed growth in a 4 % NaCl solution after 5 days of incubation and all the strains produced hydrogen sulphide (H_2S). These two characteristics need to be further investigated to determine their role in food spoilage, while the possibility of using salt to preserve food products should also be looked at.

7.4 '*Chryseobacterium piscium* sp. nov.'

In Chapter 5 a second new species, '*Chryseobacterium piscium* sp. nov.' was characterised. This proposed species comprised four strains and were obtained from marine fish, with R-23621 as the type strain. Phylogenetically, 16S rRNA sequencing of strain R-23621 showed 99.3 % similarity to *C. balustinum,* 98.9 % to *C. scophthalmum* and 97.4 % to *C. indoltheticum.* DNA-DNA hybridisations with these three reference strains showed binding values of 57 %, 51 % and 52 % respectively. The G + C mol % of the type strain is 33.6 %. This group tends to be more psychrotrophic since they grow within 24 hours at 4 °C but hardly at all at 32 °C. They are also capable of growth in a 5 % NaCl solution. In terms of phenyl alanine production, *'C. piscium'*, *C. indoltheticum* and *E. meningoseptica* strains tested positive, whereas the majority of *Chryseobacterium* species are usually negative for this trait.

7.5 Potential source of contamination

As flavobacterial strains are often present in or on stored food, it was necessary to estimate a potential source of contamination. Since the majority of strains were from chicken, different processing stages in a broiler processing plant were sampled. Literature often refers to the role of flavobacteria together with the pseudomonads in food spoilage (García-López *et al.,* 1998, 1999; Forsythe, 2000; Jay, 2002, Gram *et al.*, 2002). Undesirable flavours and odours, possible slime production and/or toxic metabolic end products are detrimental and apart from the economical loss to industry and consumers, also may have a possible health

impact on consumers. While flavobacteria have been associated with the spoilage of food, the incidence and role of flavobacteria in food deterioration is difficult to determine. They are, however, accepted as common contaminants of protein-rich foods under refrigerated storage (García-López *et al.*, 1999). A significant increase of psychrotrophic spoilage bacteria during processing is due to contamination from the air, water, handling practices and intestines. These bacteria in the end contribute to the spoilage of poultry during refrigerated storage (Hinton *et al.,* 2004).

During this study it was evident that the washing steps lowered the total counts on the carcasses, but contamination by *Chryseobacterium* species still contributed to 8.3 % of the total bacterial count on whole birds. The washing procedures after the scalding process reduced the microbial contamination significantly ($p < 0.05$), in terms of the total counts on birds and total *Chryseobacterium* counts). This was in spite of the intestinal contamination, that was expected to contribute to higher total counts of whole birds. At two different cutting stages, after the evisceration process and at the portioning line, a significant increase in the total bacterial count, as well as the total *Chryseobacterium* count took place with *Chryseobacterium* species representing between 20 and 25 % of the total population.

It can finally be concluded that the majority of Gram-negative flavobacterial strains isolated from raw chicken, red meat and fish were members of the genus *Chryseobacterium.* Apart from potential new species other identified isolates were *C. balustinum, C. defluvii, C. scophthalmum* and *C. gleum.*

Two new *Chryseobacterium* species have been proposed in this study namely; '*C. vrystaatense* sp. nov.' and '*C. piscium* sp. nov.'.

There was a significant increase in total bacterial counts and total *Chryseobacterium* counts in the processing waters during processes where portioning of the carcasses was involved.

7.6 Recommendations for future research

Further investigation need to determine whether the higher numbers of *Chryseobacterium* species on poultry, especially at the portioning stages, are caused by growth during processing or whether the bird itself, for example via the birds internal fluids, may be responsible for this contamination. Therefore it is recommended that broilers, as source of *Chryseobacterium* contamination, be investigated in future studies.

As yellow pigmented bacterial isolates colonies tend to be present on the meat and fish examined in this study, a similar study should be conducted on the Gram-positive yellow pigmented organisms in order to determine their incidence in other food commodities.

The role and significance of *Chryseobacterium* species in food spoilage should be investigated in more detail.

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SUMMARY

Microbial diversity in a food complicates the prediction or measurement of the effect of specific micro-organisms on a perishable food commodity stored under different conditions. Gram-negative yellow-pigmented colonies belonging to the *Flavobacteriaceae* family are often present when total numbers on food samples are investigated. Changes in the taxonomy of flavobacteria since its inception in 1923 have, however, complicated the identification of these bacteria. Many organisms previously regarded as *Flavobacterium* have been found to belong to several new genera in the family *Flavobacteriaceae*. With the introduction of a chemotaxonomic approach and molecular techniques such as rRNA sequencing, it is now possible to refine the differentiation between closely related genera. Chromosomal DNA characteristics further enhance accurate differentiation between various species. This study used a polyphasic taxonomic approach that included a combination of phenotypic and genotypic testing methods in order to characterise and classify Gram-negative yellow-pigmented strains isolated from raw chicken, red meat and fish.

During this study 129 Gram-negative yellow-pigmented pure cultures were isolated from raw chicken, red meat and fish samples. Protein fingerprint profiles and long chain fatty acid analyses revealed that the majority of organisms belonged to the genus *Chryseobacterium.* The 16S rRNA sequence and DNA-DNA hybridisation methods were used to give the relationship between test strains and the reference strains of various species in the genus. Several isolated strains from the chicken, red meat and fish could be equated with *C. balustinum* (2), *C. defluvii* (16), *C. scophthalmum* (4) or *C. gleum* (2) strains. A few strains (9) did not show any affiliation with any reference strain. Two new *Chryseobacterium* species, however, have been proposed from the findings of this study namely *Chryseobacterium vrystaatense* sp.nov.,which is comprised of the type strain R-23566 (= LMG 22846^{T}) and 36 strains isolated from raw chicken samples. Strains showed strong DNase- and also urease and lecithinase activity but were not capable of starch and tyrosine hydrolysis. The mol % G + C of R-23566 was 37.6 %. The second new species, *Chryseobacterium piscium* sp. nov., comprised four strains which were obtained from marine fish, with R-23621 as the type strain. The G + C mol

% was 33.6 %. This group was psychrotrophic in that they grew within 24 hours at 4 °C but very poorly at 32 °C.

In order to establish the source of potential *Chryseobacterium* contamination, different areas and processing stages in the chicken abattoir were sampled. *Chryseobacterium* species contributed to 8.3 % of the total bacterial count on whole birds in this study. It was evident that the washing processes lowered the total counts on carcasses. A significant increase in the total bacterial counts, as well as total *Chryseobacterium* counts was reported and *Chryseobacterium* species were found to represent between 20 and 25 % of the total population after portioning procedures during this study. Live chickens as source of *Chryseobacterium* contamination should be investigated in future.

Key words: *Flavobacteriaceae, Chryseobacterium*, isolation, identification, taxonomy, *C. vrystaatense* sp. nov., *C. piscium* sp. nov., sources.

OPSOMMING

Mikrobiologiese verskeidenheid in voedsel kommoditeite bemoeilik die voorspelling of meting van die uitwerking wat spesifieke mikro-organismes, het tydens opberging van bederfbare voedsel onder verskillende toestande. Gram-negatiewe geel gepigmenteerde kolonies, wat tot die familie *Flavobacteriaceae* behoort, is dikwels teenwoordig op agar-plate van totale bakterietellings van voedselmonsters. Veranderinge in die taksonomie van flavobakterieë sedert 1923, het identifikasie van hierdie bakterieë gekompliseer. Verskeie voorheen erkende *Flavobacterium* species behoort nou aan ander genera in die familie *Flavobacteriaceae.*

Sedert die toepassing van ‘n chemotaksonomiese aanslag en die ontwikkeling van meer molekulêre mikrobiologiese tegnieke soos die rRNS sekwensies, was dit moontlik om onderskeid tussen nouverwante genera te tref en DNS eienskappe het akkurate onderskeiding tussen vervante spesies moontlik gemaak. Tydens hierdie studie is daar van ‘n polifasiese taksonomiese aanslag gebruik gemaak wat ‘n kombinasie van fenotipiese sowel as genotipiese tegnieke ingesluit het, ten einde geelgepigmenteerde, Gram-negatiewe isolate wat van hoender, rooivleis en vis verkry is, te klassifiseer.

Tydens die studie is 129 Gram-negatiewe geel pigementbevattende reinkulture vanaf rou hoender-, vleis- en vismonsters geïsoleer. Proteien vingerafdrukprofiele en langkettingvetsuurontledings is gedoen, wat uitgewys het dat die meerderheid van die organismes aan die genus *Chryseobacterium* behoort. Die 16S rRNS sekwensie en die DNS-DNS hibridisasie metodes het die verwantskap tussen die isolate en die verwysingsstamme aangetoon. Etlike isolate kon as *C. balustinum* (2), *C. defluvii* (16), *C. scophthalmum* (4) of *C. gleum* (2) geidentifiseer word. ‘n Paar stamme (9) het geen noue verband met enige van die verwysingstamme getoon nie.

Twee nuwe *Chryseobacterium* spesies is in die loop van die studie voorgestel naamlik ‘*Chryseobacterium vrystaatense* sp.nov.’ , bestaande uit R-23566 (= LMG 22846^T) as verteenwoordigende stam, en 36 nouverwante isolate wat vanaf rou

hoender geïsoleer is. Hierdie stamme vertoon sterk DNase-, asook urease- en lesitinase-aktiwiteit maar geen stysel- of tirosienhidrolise nie. Die mol % G + C van R-23566 was 37.6%. Die tweede nuwe spesies, '*Chryseobacterium piscium* sp. nov.', bestaan uit vier isolate, afkomstig van seevis met R-23621 as die verteenwoordigende stam. Die G + C mol % was 33.6 %. Hierdie groep is meer psigrotrofies as die ander deurdat kolonies binne 24 ure by 4 °C gevorm word terwyl daar weinig groei by 32 °C voorkom.

Ten einde die bron van moontlike kontaminasie gedurende prosessering te bepaal, is verskillende areas en prosesseringsstappe in die hoender abattoir bemonster. C*hryseobacterium* spesies verteenwoordig 8.3 % van die totale aantal organismes op hoenderkarkasse. Dit was duidelik dat die wasprosesse totale tellings op karkasse verminder. 'n Betekenisvolle toename in die totale bakterie telling, asook die totale *Chryseobacterium*-telling, wat 20 en 25 % van die totale populasie op hoenderkarkasse verteenwoordig het, het na opsnyprosesse voorgekom. Lewende braaikuikens, as bron van *Chryseobacterium*-kontaminasie, moet in die toekoms ondersoek word.

Trefwoorde: *Flavobacteriaceae, Chryseobacterium*, isolasie, identifikasie, taksonomie, *C. vrystaatense* sp. nov., *C. piscium* sp. nov., bronne.

APPENDIX A

Article as accepted for publication in the IJSEM

Chryseobacterium vrystaatense sp. nov., isolated from raw chicken in a chicken processing plant

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Running title: *Chryseobacterium vrystaatense* sp nov., isolated from raw chicken

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The EMBL accession number for the 16S rRNA gene sequences of *Chryseobacterium vrystaatense* sp. nov. LMG 22846^T and LMG 22954 are AJ871397 and AJ871398, respectively.

Abstract

Yellow-pigmented Gram-negative organisms, isolated from raw chicken were investigated by means of a polyphasic taxonomic approach and were shown to represent a novel species in the genus *Chryseobacterium,* for which the name *Chryseobacterium vrystaatense* is proposed. Its nearest phylogenetic neighbours were *Chryseobacterium joostei, Chryseobacterium indologenes* and *Chryseobacterium gleum,* which showed 16S rRNA gene sequence similarity levels of 96.9%, 97.1% and 96.1%, respectively. The DNA-DNA hybridisation levels between strains of *C. vrystaatense* and *Chryseobacterium* reference species were below 46%. Strain LMG 22846^T (= CCUG 50970^T) was chosen as the type strain and has a mol % G+C of 37.1%.

Flavobacteria and pseudomonads are traditionally known to cause spoilage in food and food products (Forsythe, 2000). In literature on meat spoilage the genus name *Flavobacterium* is used as a generic name for yellow pigmented rods (Hendrie *et al*., 1969). Usually pathogens like *Salmonella* and *Campylobacter* are associated with poultry*,* but large numbers of other bacteria often associated with spoilage are found on poultry carcasses. These include many so-called flavobacteria that may originate from the poultry itself or from the abattoir environment and which are responsible for spoilage (Hang'ombe *et al*., 1999). Mai and Conner (2001) found that the incidence of *Pseudomonas* and flavobacteria on chicken carcasses was 17% and 16%, respectively. The incidence of flavobacteria on poultry is much higher than on other fresh meat (Nychas & Drosinos, 1999).

Over the past decade many changes have taken place in the taxonomy of the family *Flavobacteriaceae* and the revised genus *Flavobacterium* presently comprises mainly aquatic bacteria that are not known in food microbiology (Bernardet *et al*., 1996). Several former *Flavobacterium* species were transferred to the novel genus *Chryseobacterium* (Vandamme et al., 1994) and novel species including *Chryseobacterium joostei* (Hugo *et al*., 2003)*, Chryseobacterium defluvii* (Kämpfer *et al*., 2003), *Chryseobacterium miricola* (Li *et al.,* 2003), and, most recently, *C. formosense* (Young *et al*., 2005) *C. daecheongense* (Kim *et al*., 2005a) and *C. taichungense* (Shen *et al*., 2005) have been reported. "*Chryseobacterium proteolyticum"* was described by Yamaguchi & Yokoe (2000), but its name has not been validated. Also, two *Chryseobacterium* species, *C. meningosepticum* and *C. miricola,* have been reclassified into the new genus, *Elizabethkingia* (Kim *et al*., 2005b). In general, these *Chryseobacterium* species are

widely distributed in water, soil, and the clinical environment, but also in food specimens, such as milk, meat, poultry and fish (Jooste & Hugo, 1999).

During a study on the incidence of yellow-pigmented strains isolated from meat, a total of 36 yellow-pigmented isolates were obtained from raw chicken, at different stages of processing, from a local chicken processing plant in the Vrystaat province (Free State, South Africa). Table A in the on-line version of the manuscript contains information on the investigated isolates, their origin and number. The spoilage ability of this group is not yet determined. *Chryseobacterium* reference strains were available from the BCCM/LMG Bacteria Collection. After growth for 24 h on Tryptic Soy agar (BBL), cells were harvested and whole-cell protein extracts were prepared and subjected to whole-cell protein electrophoresis as described by Pot *et al.* (1994). A densitometric analysis, normalization and interpolation of the protein profiles, and a numerical analysis were performed by using the Gelcompar software package (Applied Maths). The 36 isolates formed 3 subgroups comprising 19, 4 and 13 isolates, respectively (see Fig. A in Supplementary information) available in the online version of this paper. Isolates belonging to different subgroups differed mainly in the density of some protein bands (Fig. A).

High-molecular-weight DNA was prepared according to Pitcher *et al.* (1989) from a representative isolate of each of these three subgroups, i.e. isolates LMG 22846^T, LMG 22848 and LMG 22847, and from *Chryseobacterium* reference strains (see below). DNA-DNA hybridisations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridisation temperature was 33 °C. The hybridisation values obtained among the three isolates were above 92 %, demonstrating that the three subgroups formed a homogeneous genospecies.

In order to determine the phylogenetic position of this taxon, the 16S rRNA gene sequence of LMG 22846^T and of LMG 22954 was determined as described by Willems *et al.* (2003). The 16S rRNA sequence of LMG 22846^T and LMG 22954 showed 99.9 % similarity to each other and 96.9 %, 97.1 % and 97.1 % similarity to *C. joostei, C. indologenes* and *C. gleum,* respectively, demonstrating that this taxon belonged in the genus *Chryseobacterium.* Values towards the other type strains were below 96%. Fig. 1 illustrates the phylogenetic position of this taxon within the *Chryseobacterium* genus. Subsequent DNA-DNA hybridisations towards the type strains of the latter three species yielded a value of 46 % between strain LMG 22846^T and the *C. joostei* type strain; all other values were between 27 and 32%.

In order to determine the mol % G+C, DNA was enzymatically degraded into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by HPLC using a Waters SymmetryShield C8 column regulated at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ pH 4.0 with 1.5 % acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. The detector used was a Waters model 484 UV-VIS absorbance detector set at 270 nm. The mol % G + C of strain LMG 22846^T, LMG 22847 and LMG 22848 was 37.1, 37.0 %, and 37.6 %) respectively, which is in the range of 34 -38 mol % for the *Chyseobacterium* genus (Vandamme *et al.*, 1994).

The whole-cell fatty acid components of seven strains representing different groups and of *Chryseobacterium* reference strains were prepared from a loopful well-grown cells incubated for 24 h at 28 ˚C. Separation and identification of esters were done with the Sherlock Microbial Identification System (MIDI version 3.0) as previously described by Vandamme *et al.* (1992). Mean percentages and standard deviations were calculated and are shown in Table 1. As for other *Chryseobacterium* species, the predominant fatty acids were C15:0 iso (40 – 43 %), C17:1 iso ω9c (16 – 21 %) and C17:0 iso 3-OH (15 – 18 %).

A detailed phenotypic characterization of the 36 isolates and *Chryseobacterium* reference species was subsequently performed. A 24 h culture, incubated at 25 °C, was suspended in a quarter strength Ringer solution to give a McFarland Barium Sulphate Standard 2 suspension. A battery of tests was selected to differentiate the species in the genus at 25 °C according to the methods described by Cowan (1974), MacFaddin (1980), Gerhardt *et al.* (1981) and Hugo *et al.* (2003). Twenty-two isolates were subjected to the Biolog GN2 MicroPlate (Biolog, Inc., Hayward, Calif.) according to the manufacturer's protocol. Test results are shown below in the species description and in Table 2 which includes the biochemical tests useful for the differentiation of the novel species from established *Chryseobacterium* species.

The results of the present study demonstrate that the collection of 36 yellow-pigmented isolates from raw chicken represent a novel species in the genus *Chryseobacterium* for which we propose the name *Chryseobacterium vrystaatense*.

Description of *Chryseobacterium vrystaatense* sp. nov.

Chryseobacterium vrystaatense (vry.sta.'ten.se, N. L. neutr. adj., named after Vrystaat (Free Sate), the South African province where these bacteria were isolated).

Colonies are shiny, orange-yellow and translucent with entire edges. Cells are Gram-negative, catalase and oxidase positive and produce flexirubin type pigments, they are non-motile and neither oxidative nor fermentative. Colonies are formed after 24 h at 4, 15, 25 and 32 °C on nutrient agar but no growth occurs at 37 and 42 °C. Growth on MacConkey agar no. 3 (Oxoid CM115) occurs after 48 h at 15, 25 and 32 °C for all but one strain that only grew on MacConkey agar at 32 °C after 48 h. All strains grow in nutrient broth containing 1 and 2 % NaCl (w/v); growth in a 3 % NaCl-broth is strain-dependent (14 strains – excluding the type strain – tested positive for growth). Although several strains showed growth at different salt concentrations initially, only 5 strains showed growth in 4 % NaCl-broth after 5 days incubation (R-23493, R-23569, R-23575, R-23576 and R-23595). Strains show strong DNase activity and also urease and lecithinase activity but no starch and tyrosine hydrolysis. Reaction on triple sugar agar (Oxoid CM277) and 10 % lactose are alkaline. Ability to produce H_2S varies depending on incubation time on triple sugar agar and SIM medium (Oxoid CM435). Both media contain sodium thiosulphate but SIM medium also contains ferrous ammonium sulphate and more than 50% of strains gave a positive reaction.

The following positive reactions were encountered in the BIOLOG system for all the strains tested (n = 22): Tween 40 and 80, gentobiose, α-D-glucose, D-mannose, D-trehalose, succinic acid mono-methyl esther, acetic acid and L-asparagine. In the case of glycyl-L-glutamic acid, glycerol, L-glutamic acid, L-serine and L-aspartic acid, at least 19 strains (85 %) reacted positively. Reactions in which more than 50 % of strains give a positive reaction, were dextrin (16+), D-mannitol (18+), α-ketovaleric acid (15+), L-alanyl-glycine (12+), L-threonine (16+), inosine (13+), uridine (15+) and thymidine (12+).

The type strain is LMG 22846^T (= CCUG XXX ^{T}) and has been isolated from a raw chicken portion sample obtained from a chicken processing plant. The other (35) isolates were obtained from raw chicken samples, collected at different processing stages (portion, whole bird, automatic line, manual line and the spiral freezing process on different occasions. Three additional isolates have been deposited in the BCCM/LMG Bacteria Collection as LMG 22847, LMG 22848, and LMG 22954.

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Table 1. Long chain fatty acid composition of *Chryseobacterium* species

Taxa are listed as: 1, *C. vrystaatense* sp. nov. (n=7); 2, *C. indoltheticum* (n=1); 3, *C. balustinum* (n=1); 4, *C. gleum* (n=5); 5, *C. indologenes* (n=45); 6, *C. scophthalmum* (n=2); 7, *C. joostei* (n=11); 8, *C. defluvii* (n=1); 9, *C. formosense* (n=1); 10, *C. daecheongense* (n=1); 11, *C. taichungense* (n=1); 12, *Elizabethkingia meningoseptica* (n=1); 13, *Elizabethkingia miricola* (n=1). Fatty acid percentages amounting to less than 1.0 % of the total fatty acids in all strains were not included. Trace (tr) for values less than 1%; ND, not detected; ECL, equivalent chain length (i.e. the identity of the fatty acids is unknown). Means ± SD are given for *C. vrystaatense* only. Data from Hugo *et al.*, 2003 (2-7, 12), Li *et al.*, 2003 (13), Young *et al.* 2005 (9), Kim *et al.,* 2005a (10), Shen *et al.*, 2005 (11).

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13
C13:0 iso	1.1±0.4	ND	tr	tr	tr	tr	tr	2.8	3.6	1.6	tr	1.4	tr
ECL 13.566	1.4±0.3	1.7	1.6	1.2	2.1	2.9	1.1	tr	tr	1.5	6.7	1.5	tr
C15:0 anteiso	1.7±0.7	5.9	tr	tr	tr	tr	tr	3.2	2.1	1.0	tr	2.3	tr
C15:0 iso	41.8±1.4	29.4	32.3	35.4	34.3	35.0	34.6	58.5	52.2	51.2	35.4	41.4	33.0
C15:0 iso 3-OH	2.7±0.3	2.3	2.7	2.5	2.6	2.7	2.9	2.6	1.8	2.0	4.3	3.5	3.0
C16:0	1.1±0.3	1.0	1.6	1.3	tr	1.2	tr	1.3	1.5	1.8	1.3	tr	2.0
C16:0 3-OH	1.3±0.3	tr	1.4	1.1	1.0	1.0	1.2	tr	tr	tr	2.6	2.2	tr
C16:0 iso 3-OH	tr	1.3	tr	ND	ND	ND	ND	tr	1.1	tr	1.4	tr	8.0
ECL 16.580	1.2±0.2	1.3	1.3	1.7	1.7	1.5	1.6	tr	1.0	1.0	1.7	1.7	tr
C17:0 iso	tr	tr	1.0	1.6	tr	tr	tr	2.0	2.3	3.0	tr	tr	1.0
C17:0 iso 3-OH	15.4±1.8	14.0	16.8	21.8	19.2	16.3	20.1	14.1	10.9	15.7	22.4	16.3	40.0
C17:1 iso ω9c	19.7±2.3	25.6	27.1	20.2	24.2	24.8	22.9	4.8	4.3	7.6	8.9	7.0	tr
C18:1 ω5c	tr	tr	tr	tr	tr	tr	tr	NA	ND	ND	ND	tr	NA
Summed feature 3[#]	9.1±0.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Summed feature 4[#]	tr	11.2	9.2	11.8	11.1	11.5	12.1	8.4	6.5	8.4	13.8	18.1	12
Summed feature 5[#]	NA	ND	tr	tr	tr	ND	tr	NA	ND	ND	ND	tr	NA

Summed feature 3 comprises 16:1 ω7c and/or 15:0 iso 2-OH; Summed feature 4 comprises 15:0 iso 2-OH and 16: 1 ω7ct; Summed feature 5 comprises 17:1 iso I and/or 17:1 anteiso B.

Table 2. Phenotypic properties that differentiate *C. vrystaatense* sp. nov. from other *Chryseobacterium* species

Taxa are listed as: 1, *C. vrystaatense* sp. nov. (n=36); 2, *C. indoltheticum* (n=1); 3, *C. balustinum* (n=1); 4, *C. gleum* (n=1); 5, *C. indologenes* (n=1); 6, *C. scophthalmum* (n=1); 7, *C. joostei* (n=1); 8, *C. defluvii* (n=1); 9, *C. formosense* (n=1); 10, *C. daecheongense* (n=1); 11, *C. taichungense* (n=1); 12, *Elizabethkingia meningoseptica* (n=1); 13, *Elizabethkingia miricola* (n=1); Data from Li *et al.*, 2003 (13), Young *et al.* 2005 (9), Kim *et al.,* 2005a (10), Shen *et al.*, 2005 (11). +, Positive; w, weakly positive; -, negative; D, delayed; NA, not available; #, number of strains tested positive including type strain.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13
Growth on/at:													
Cetrimide agar	+[a]	-	-	-	+	-	+	-	NA	NA	NA	+	+
MacConkey agar	-[b]	+	+	+	+	-	+	-	-	-	-	+	+
Growth on Nutrient agar:													
5˚C	+	+	+	-	-	+	+	-	-	-	-	-	NA
37°C	-	-	+	+	+	-	-	+	-	+	+	+	-
42°C	-	-	-	-	+	-	-	+	-	-	-	+	NA
3% NaCl	-	w	w	+	+	w	w	-	NA	NA	NA	w	NA
Enzyme activity:													
Urease	+	-	-	-	-	+	+	-	-	-	-	D	+
Lecithinase	+	+	+	+	+	+	+	+	NA	NA	NA	-	+
Nitrate reduction	-	-	+	+	-	-	-	-	-	+	NA	-	-
Nitrite reduction	+	-	-	+	-	-	-	-	-	-	NA	+	-
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	NA	+	+
Starch hydrolysis	-	-	-	+	+	-	+	w	NA	+	NA	-	-
Tween 80 hydrolysis	+	+	+	+	+	+	+	NA	-	-	NA	-	+
Tyrosine hydrolysis	-	D	-	+	-	+	+	+	NA	NA	NA	D	-
Production of:													
Hydrogen sulphide	23[#]	+	-	-	-	-	-	-	-	-	-	-	+
Indole	+	+	+	+	+	-	w	w	+	-	w	D	+

Variable reactions are scored as: *a*, positive for all strains except R-23519, R-23533, R-23598; *b*, majority of strains were negative although some grow after 48 h at 25°C.

Table A (on-line version of the manuscript). Strains investigated and their origin

Abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratory for Microbiology, University of Ghent, Belgium. R, research collection.

Strain	Origin
***Chryseobacterium vrystaatense* sp. nov.**	
R-23482	Chicken portion, 25/06/2002
R-23483	Chicken portion, 25/06/2002
R-23493	Chicken portion, 10/03/2003
LMG 22848 (= R-23500)	Chicken portion, 10/03/2003
R-23502	Chicken portion, 10/03/2003
R-23510	Whole bird, 17/03/2003
R-23511	Whole bird, 03/05/2003
R-23515	Whole bird, 03/05/2003
R-23517	Whole bird, 03/05/2003
R-23519	Whole bird, 03/05/2003
R-23529	Whole bird, 03/05/2003
R-23532	Manual line, 03/05/2003
LMG 22847 (= R-23533)	Spiral freezing process, 03/05/2003
R-23554	Automatic line, 18/02/2002
R-23559	After spin chiller, 24/02/2002
R-23562	Whole bird, 24/02/2002
R-23563	Spiral freezing process, 24/02/2002
R-23564	Spiral freezing process, 24/02/2002
R-23565	Spiral freezing process, 24/02/2002
LMG 22846^T (= R-23566^T)	Portion, 24/02/2002
R-23569	Whole bird, 24/02/2002
R-23571	Whole bird, 24/02/2002
R-23572	After spin chiller, 10/02/2002
R-23574	After spin chiller, 10/02/2002
R-23575	After spin chiller, 10/02/2002
R-23576	After spin chiller, 10/02/2002
R-23579	Chicken portion, 10/02/2003
R-23580	Chicken portion, 10/02/2003
R-23583	Spiral freezing process, 10/02/2003
R-23588	Automatic line, 10/02/2003

R-23589	Automatic line, 10/02/2003
R-23594	Manual Cutting Process, 10/02/2003
R-23595	Manual Cutting Process, 10/02/2003
R-23598	Chicken portion, 03/03/2003
LMG 22954 (= R-23600)	Chicken portion, 03/03/2003
R-23601	Chicken portion, 03/03/2003
C. indoltheticum LMG 4025^T	Marine mud, 1977
C. balustinum LMG 8329^T	Fish, heart blood, 1988
C. gleum LMG 8334^T	Vaginal swab, London, 1982
C. indologenes LMG 8337^T	Human trachea at autopsy, USA, 1971
Elizabethkingia (*Chrys.*) *meningoseptica* LMG 12279^T	Infant cerebral fluid, USA, 1958
C. scophthalmum LMG 13028^T	Diseased turbot gills, Scotland, 1987
C. joostei LMG 18212^T	Raw cow milk, South Africa, 1981
C. defluvii LMG 22469^T	Activated sewage sludge, Germany
Elizabethkingia (*Chrys.*) *miricola* LMG 22470^T	Condensation water, space station Mir, 1997
Empedobacter brevis LMG 4011^T	Human bronchial secretions, Switzerland, 1977
Bergeyella zoohelcum LMG 8351^T	Human sputum, Nebraska, U.S.A, 1982
Weeksella virosa LMG 12995^T	Human urine, 1992
Myroides odoratus LMG 1233^T	Patent strain, 1976
Flavobacterium aquatile LMG 4008^T	Deep well, Chalk region Kent, U.K., 1978
Sphingobacterium spiritivorum LMG 8347^T	Intra-uterine specimen, 1983

1%

Elizabethkingia miricola LMG 22470^T (AB071953)
Elizabethkingia meningoseptica LMG 12279^T (AJ704540)
Kaistella koreensis KCTC 12107^T (AF344179)
Sejongia jeonii IMSNU 14049^T (AY553294)
Chryseobacterium formosense CC-H3-2^T (AJ715377)
Chryseobacterium defluvii LMG 22469^T (AJ309324)
Chryseobacterium daecheongense KCTC 12088^T (AJ457206)
"*Chryseobacterium proteolyticum*" 9670 (AB039830)
Chryseobacterium taichungense TWGS-8^T (AJ843132)
Chryseobacterium indologenes LMG 8337^T (M58773)
Chryseobacterium gleum LMG 8334^T (M58772)
Chryseobacterium joostei LMG 18212^T (AJ271010)
Chryseobacterium vrystaatense LMG 22846^T (AJ871397)
Chryseobacterium vrystaatense LMG 22954 (AJ871398)
Chryseobacterium indoltheticum LMG 4025^T (M58774)
Chryseobacterium balustinum LMG 8329^T (M58771)
Chryseobacterium scophthalmum LMG 13028^T (AJ271009)
Ornithobacterium rhinotracheale LMG 9086^T (L19156)

Fig. 1. Neighbour joining phylogenetic tree of *C. vrystaatense* and related bacteria based on 16S rRNA sequence comparisons. Bootstrap values above 60% obtained with 1000 repetitions are indicated. The 16S rDNA sequence of *Ornithobacterium rhinotracheale* LMG 9086^T was included as outgroup.

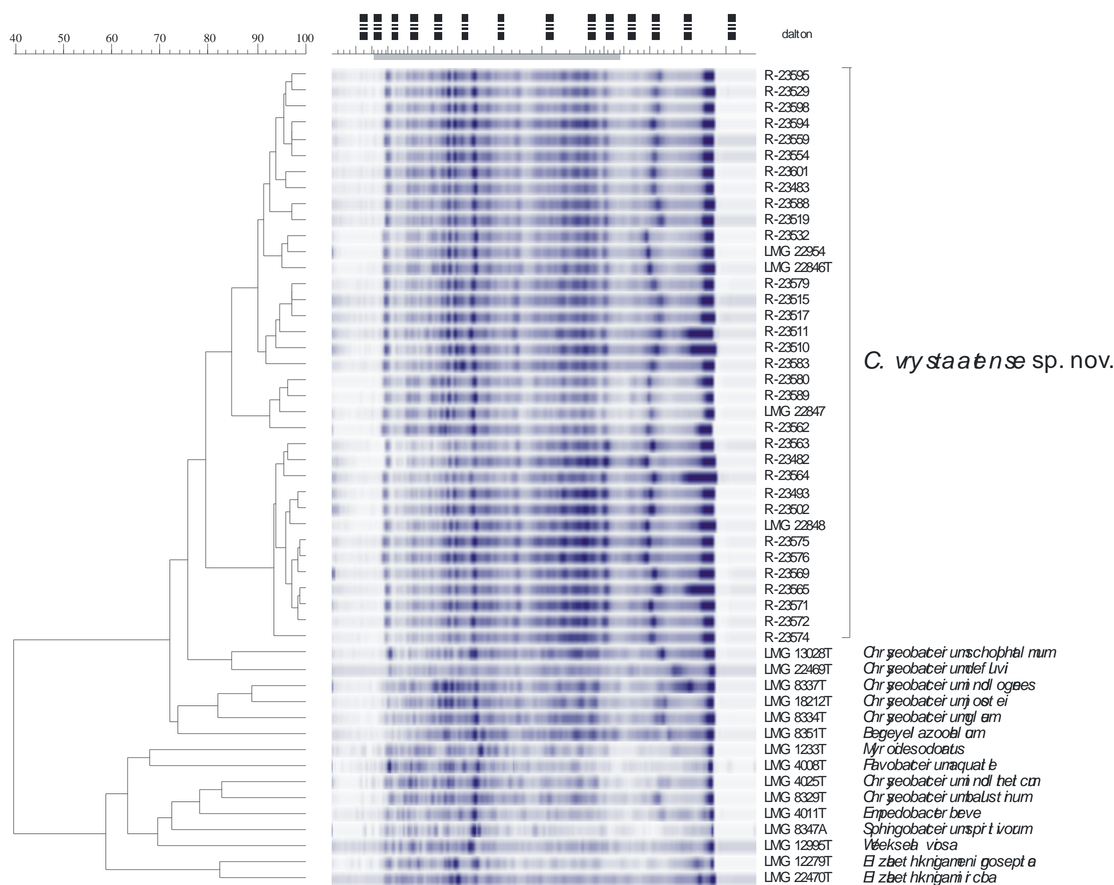

Fig. A. Dendrogram derived from the unweighted pair group average linkage of correlation coefficients between the whole-cell protein profiles of *C. vrystaatense* and reference strains of related taxa. Protein profiles were reproduced using the GelCompar software package. The correlation coefficient is expressed as percentage similarity for convenience. The grey bar in the marker lane indicates the part of the protein profile that was used in the numerical analysis.