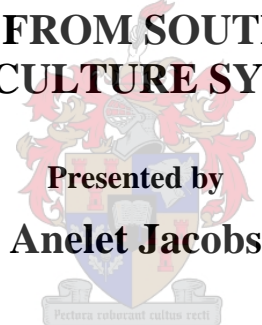




University of Stellenbosch

**INVESTIGATION AND COMPARISON OF
ADHERENCE- AND BIOFILM-FORMING CAPACITIES
OF YELLOW-PIGMENTED *CHRYSEOBACTERIUM*,
ELIZABETHKINGIA AND *MYROIDES* SPP.
ISOLATED FROM SOUTH AFRICAN
AQUACULTURE SYSTEMS**



**Presented by
Anelet Jacobs**

Thesis presented in partial fulfillment of the requirements for the degree of

Masters of Science in Microbiology

In the department of Microbiology at the University of Stellenbosch

**Supervised by Dr. H.Y. Chenia
Co-supervisor: Prof. D.E. Rawlings**

March 2007



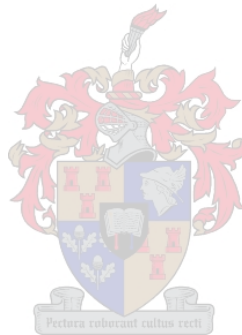
DECLARATION

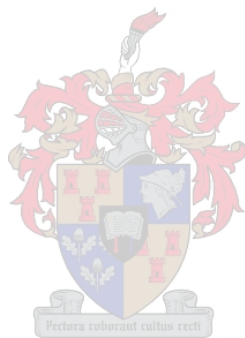
I, the undersigned, hereby declare that the work contained in this thesis is my own, unaided work. It has been submitted for the degree of Master of Science in the University of Stellenbosch. It has not been submitted previously, in its entirety or in part, at any other university.

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ABSTRACT

In the aquaculture setting, opportunistic pathogens are present as part of the normal aquatic microflora, colonizing surfaces in fish tanks as part of biofilm communities, and often causing severe economic losses to the aquacultural industry. Isolates belonging to the genera *Chryseobacterium*, *Elizabethkingia*, *Myroides* and *Empedobacter* have been isolated from diseased fish, and are responsible for causing secondary fish infections, fish- and food-product spoilage, and have been described as etiological agents of various human diseases. Thirty-four *Chryseobacterium* and *Elizabethkingia* spp. and five *Myroides* and *Empedobacter* spp. isolates, obtained from various diseased fish species and biofilm growth in South African aquaculture systems, were characterised genetically using 16S rRNA gene PCR restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) PCR, whole cell protein (WCP) and outer membrane protein (OMP) analyses. Genetic heterogeneity was displayed by the *Myroides* and *Empedobacter* spp. study isolates following OMP analysis, although 16S rRNA gene RFLP, RAPD-PCR and WCP analysis did not allow for differentiation of these isolates. A high degree of genetic heterogeneity was displayed by the *Chryseobacterium* and *Elizabethkingia* spp. study isolates following OMP analysis, 16S rRNA gene RFLP with *MspI*, and RAPD-PCR with primer P2. However, based on the results obtained by WCP analysis, 16S rRNA gene RFLP with *CfoI* and *TaqI*, and RAPD-PCR with primer P1 the isolates appeared genetically very homogeneous. High MAR indices and potential multi-drug resistance phenotypes were obtained for the *Myroides* and *Empedobacter* spp. and some of the *Chryseobacterium* and *Elizabethkingia* spp. isolates by antimicrobial susceptibility testing. Primary adherence and the influence of environmental changes on adherence was investigated by a modified microtitre-plate adherence assay. Nutrient composition, temperature and hydrodynamic incubation conditions were observed to influence adherence abilities of all study isolates. In addition, adherence varied greatly among isolates of the genera *Chryseobacterium* and *Elizabethkingia*, as opposed to a consistent strong adherence profile observed for the *Myroides* and *Empedobacter* spp. isolates. The influence of cell surface properties such as capsule presence and cell surface hydrophobicity, on primary adherence of the isolates was also investigated. Quantitative analysis of capsular material revealed the presence of

thick capsular material surrounding the *Myroides* and *Empedobacter* spp. and some of the *Chryseobacterium* and *Elizabethkingia* spp. isolates, but could not be directly associated with adherence. Hydrophobicity were investigated using the salt aggregation assay (SAT) and bacterial adherence to hydrocarbon test (BATH). A very hydrophilic cell surface was observed for all of the *Myroides* and *Empedobacter* spp. isolates, and majority (74%) of the *Chryseobacterium* and *Elizabethkingia* spp. isolates. Cell surface hydrophobicity could not be correlated to the adherence of the *Myroides* and *Empedobacter* spp. isolates, and only SAT-determined hydrophobicity could be positively correlated to adherence of *Chryseobacterium* and *Elizabethkingia* spp. isolates under certain conditions. Coaggregation studies were performed between the study isolates and various important clinical and aquacultural microorganisms. High coaggregation indices were observed between the *Myroides* and *Empedobacter* spp. isolates and *E. faecalis* and *S. aureus*, and between *E. faecalis*, *S. enterica* serovar Arizonae, *S. aureus* and *Listeria* spp. and the *Chryseobacterium* and *Elizabethkingia* spp. isolates. Biofilm-forming capacity of the study isolates in an environment simulating their natural environment was investigated microscopically using a flow cell system. Typical 'cone-like' biofilm structures were observed for selected strains of both *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates. The effect of increased hydrodynamics on biofilm architecture was seen through the narrowing of the biofilm structures and the formation of single cell chains towards the increased hydrodynamic area of the flow chambers. *Chryseobacterium* and *Elizabethkingia* spp. and *Myroides* and *Empedobacter* spp. appear to be potential primary biofilm-formers associating with a variety of microbes thus perpetuating their survival in a variety of aquatic habitats.

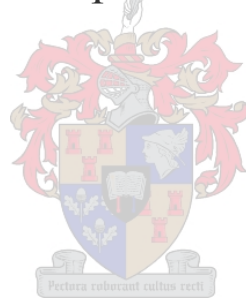
OPSOMMING

Opportunistiese patogene kom gereeld in akwakultuur sisteme voor as deel van die akwatiese mikroflora wat dikwels biofilms vorm op oppervlaktes in hierdie sisteme. Visinfeksies veroorsaak deur hierdie patogene lei tot ernstige ekonomiese verliese vir akwakultuur industrieë. *Chryseobacterium*, *Elizabethkingia*, *Myroides* en *Empedobacter* spp. is reeds voorheen van verskeie geïnfekteerde visspesies geïsoleer hierdie bakterieë is verantwoordelik vir sekondere visinfeksies, die bederf van vis- en kosprodukte, asook menslike siektes. Vier-en-dertig *Chryseobacterium* en *Elizabethkingia* spp. en 5 *Myroides* en *Empedobacter* spp. isolate, geïsoleer vanaf verskeie geïnfekteerde visspesies en biofilm-groei in Suid Afrikaanse akwakultuur-sisteme, is geneties met behulp van 16S rRNS geen PKR restriksie fragment lengte polimorfisme (RFLP), toevallig geamplifiseerde polimorfiese DNS (TGPD) PKR, heel-sel protein (HSP) en buitemembraan protein (BMP) analise gekarakteriseer. BMP analise het getoon dat die *Myroides* en *Empedobacter* spp. isolate geneties heterogeen is, alhoewel 16S rRNS TGPD-PKR, TGPD-PKR en HSP analise nie tussen die isolate kon onderskei nie. BMP analise, 16S rRNS TGPD-PKR met *MspI* en TGPD-PKR met inleier P2 was meer suksesvol as HSP analise, 16S rRNS TGPD-PKR met *CfoI* en *MspI*, en TGPD-PKR met inleier P1, om onderskeid te tref tussen die *Chryseobacterium* en *Elizabethkingia* spp. isolate en het gedui op 'n hoë vlak van genetiese heterogeniteit tussen hierdie isolate. Beide die *Chryseobacterium* en *Elizabethkingia* spp. en *Myroides* en *Empedobacter* spp. isolate het 'n hoë vlak van antibiotika weerstand getoon wat dui op 'n menigvuldigde antibiotika weerstands-fenotiepe. Primêre vashegting vermoëns en die invloed van omgewingsfaktore op vashegting is met behulp van 'n gemodifiseerde mikrotiterplaat vashegtings toets ondersoek. Vashegting van die isolate is beïnvloed deur variasies in die samestelling van die medium, temperatuurveranderinge en verskillende hidrodinamiese inkubasie kondisies. Inteenstelling met die sterk vashegtingsvermoë van die *Myroides* en *Empedobacter* spp. isolate, het die vermoë om vas te heg grootliks tussen die *Chryseobacterium* en *Elizabethkingia* spp. isolate gevarieer. Verder is ondersoek ingestel op die invloed van seloppervlak eienskappe soos die teenwoordigheid van kapsules en hidrofobisiteit op die isolate se vermoë om aan oppervlaktes te heg. Die *Myroides* en

Empedobacter spp. isolate en verskeie *Chryseobacterium* en *Elizabethkingia* spp. isolate is omring deur dik kapsules, maar geen verband tussen vashegting en die teenwoordigheid van kapsules kon bepaal word nie. Die sout aggregasie toets (SAT) en bakteriële vashegting aan koolwaterstowwe (BVAK) toets was gebruik om die hidrofobisiteit van die isolate se seloppervlaktes te bepaal. Die *Myroides* en *Empedobacter* spp. isolate en 74% van die *Chryseobacterium* en *Elizabethkingia* spp. isolate het 'n baie hidrofiliese seloppervlak getoon. Slegs die hidrofobisiteit bepaal deur die SAT toets het 'n positiewe verwantskap met die aanhegtingsvermoë van die *Chryseobacterium* en *Elizabethkingia* spp. isolate getoon. Mede-aggregasie tussen die isolate en verskeie belangrike mediese en akwakultuur mikroörganismes is ook ondersoek. Die *Myroides* en *Empedobacter* spp. isolate het 'n sterk assosiasie met *E. faecalis* en *S. aureus* getoon. Die *Chryseobacterium* en *Elizabethkingia* spp. isolate het sterk met *E. faecalis*, *S. aureus*, *S. enterica* serovar Arizonae en *Listeria* spp. geassosieer. Vloei-sel studies is uitgevoer om die biofilm-vormingsvermoë van die isolate te ondersoek. Vir beide die *Myroides* en *Empedobacter* spp. en *Chryseobacterium* en *Elizabethkingia* spp. isolate is tipiese kegelagtige biofilm stukture waargeneem. Die invloed van verhoogde hidrodinamiese kondisies in die vloei-sel het vernouing van die biofilm stukture en die vorming van enkel-sel kettings tot gevolg gehad. Vanuit hierdie studie is afgelei dat die *Myroides* en *Empedobacter* spp. en *Chryseobacterium* en *Elizabethkingia* spp. isolate onder verskeie kondisies aan oppervlaktes kan vasheg en dus potensiële primêre biofilm-vormings organismes is. Hierdie organismes besit ook die vermoë om met 'n verskeidenheid ander organismes te assosieer, wat waarskynlik hulle suksesvolle oorlewing in akwakultuursisteme verseker.

This Work Is Dedicated To

Oupa Ben



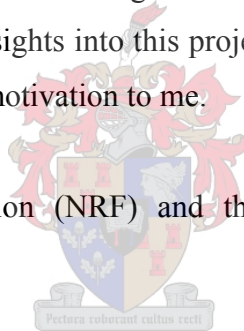


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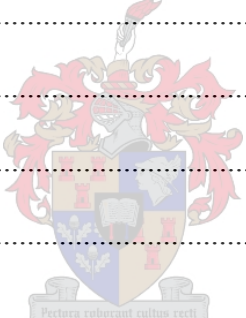
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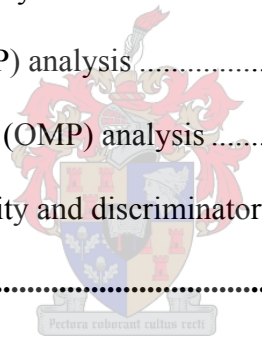
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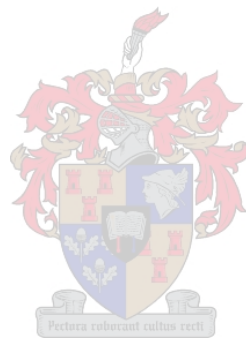
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CHAPTER ONE

Literature Review

1. Aquaculture

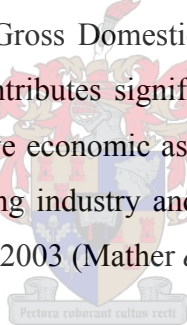
1.1 *The World*

Aquaculture has been a source of fish protein for human consumption for nearly 4000 years (Iwama, 1991). From 1984 to 1994 the world aquaculture production more than doubled, making it one of the fastest growing food production activities (FAO, 1996). Today, one fourth of the fish consumed by humans is the product of aquaculture, and this percentage will only increase as aquaculture expands and the worlds' conventional fish catch from oceans and lakes continues to decline because of over-fishing and environmental damage (Folke and Kautsky, 1992).

Aquaculture products fall into two distinct groups: high-valued species such as shrimp and salmon produced for export, and lower-valued species including carp and tilapia, which are primarily produced for local consumption. Aquaculture does not only serve as a significant contribution to the protein needs of low-income regions, but also boosts the economy of these regions through export of high-valued species to high-income regions (Christensen, 1997; and Holmes, 1996). In addition, with the production of high-valued species in low-income regions and subsequent increase in economy, aquaculture also contributes in the fight against poverty. With a growth rate of approximately 20% a year, the aquaculture industry in China, the largest aquaculture industry in the world currently employing 10 million people, promises more jobs annually. Globally, the aquacultural industry is expected to expand, as the worlds' need for fish protein is increasing. It has been predicted that by 2010, fish protein produced by aquaculture would have increased by 50% to 39 million metric tons (FAO, 1996). It can be said without a doubt, that the aquacultural industry plays a very important role in contributing to the global food supply, alleviation of poverty and survival of poor countries.

1.2 South Africa

In South Africa, the aquaculture industry includes freshwater culture and mariculture. In 2003, approximately 250 tons of trout and salmon were produced by the freshwater industry, primarily in the Western Cape. Other freshwater species cultivated on a small-scale include catfish (*Clarias gariepinus*), freshwater crayfish (maron) and tilapia species. Mariculture is the largest industry in South Africa, focusing on mussels, oysters, abalone, seaweeds and prawns. Of these, mussel farming is the best established, producing most of the estimated 2 650 tons of mariculture in 2003. Another established industry is abalone culture in the southern part of the Cape coast. Currently, there is an experimental offshore farm (cage-culture) of salmon in the Western Cape. Although the South African fisheries sector only plays a small part in the economy of the country, contributing only about 1% to the Gross Domestic Product (GDP), it has a substantial level of international trade, and contributes significantly to foreign exchange of South Africa. In addition, a comprehensive economic assessment revealed that 16 854 people were directly employed in the fishing industry and secondary and associated industries employed a further 10 876 people in 2003 (Mather *et al.*, 2003).



2. Bacterial disease as threat to aquaculture industry

The most important threat to the aquaculture industry is disease, often caused by bacteria. Another threat relating to this is the spoilage of fish products by microorganisms after harvest, and during packaging, transport and storage. Due to the above-mentioned increasing global importance of aquaculture, the study of these microorganisms is becoming increasingly important. The presence of microorganisms in aquaculture plays a very important role in the early life stages of the development of fish, since microbial colonization of the egg surface and presence of microorganisms during larval stages is detrimental to the development of gut microflora (Hansen and Olafsen, 1999).

In addition, the majority of bacteria causing disease in fish are opportunistic pathogens that are present as part of the normal water microflora (Hansen and Olafsen, 1999). These disease-causing organisms are often found colonizing surfaces of cultivation tanks, and present in or on fish cultivated for human consumption. Bacterial disease is often found to take place during stressful periods when the fish undergo physiological changes due to internal stresses such as diseases associated with spawning (Inglis *et al.*, 2001). External environmental stressors, such as changes in temperature and oxygen concentration, pollutants, or chemical and abrasive forces may also play a role in weakening the first-line defenses to allow for bacterial colonization (Hansen and Olafsen, 1999). Many bacterial species are known to infect fish, from primary pathogens such as *Vibrio anguillarum* and *Aeromonas salmonicida* to a great range of opportunistic pathogens, including *Aeromonas hydrophila*, *Vibrio harveyi*, *Yersinia ruckeri*, and members of the yellow-pigmented family *Flavobacteriaceae* (Inglis *et al.*, 2001). These fish pathogens have caused tremendous problems in the aquaculture industry worldwide (Darwish *et al.*, 2004; Gavin *et al.*, 2003; Wang *et al.*, 2003; Coquet *et al.*, 2002a; Kondo *et al.*, 2002; Decostere *et al.*, 1999; and Karunasagar *et al.*, 1996).

The various fish diseases caused by these pathogens include bacterial cold-water disease (BCWD) caused by *Flavobacterium psychrophilum*, bacterial gill disease (BGD) primarily associated with Gram-negative filamentous yellow-pigmented bacteria including *Flavobacterium columnare* and *Flavobacterium branchiophilum*, and *Flavobacterium columnare* which is the etiological agent of columnaris disease (Inglis *et al.*, 2001). Bacterial kidney disease (BKD) is caused by *Renibacterium salmoninarum* and *Edwardsiella tarda* is the causative agent of septicaemia in fish and has also been implicated in spoilage of fish products (Inglis *et al.*, 2001). *Aeromonas* spp. are involved in various diseases, often causing secondary infection, and thus described as opportunistic pathogens. Some bacterial fish pathogens such as *Psiscirickettsia salmonis* are obligate intracellular parasites of fish. Other fish pathogens include *Yersinia ruckeri*, the causative agent of enteric redmouth (ERM) disease, and *Vibrio anguillarum* and *Aeromonas salmonicida* (Inglis *et al.*, 2001).

Apart from the above-mentioned bacterial fish diseases, numerous other diseases caused by various bacterial fish pathogens and opportunistic pathogens exist. These

bacterial diseases are the most important causes of losses among fish farm stocks, therefore necessitating a full understanding for successful therapy and control to avoid severe economical aquacultural losses.

2.1 The family *Flavobacteriaceae*

The family *Flavobacteriaceae* belongs to the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB), together with several other families and many isolated taxa (Bernardet *et al.*, 2002). Gram-negative, non-spore-forming, yellow-pigmented rods that weakly produce acid from carbohydrates were initially classified into the genus *Flavobacterium* (Bernardet *et al.*, 1996). As this genus rapidly acquired a large number of poorly defined bacteria resulting in a very heterogeneous group of bacteria, two new genera were proposed. Aerobic, cellulolytic, gliding soil bacteria were reclassified into the genus *Cytophaga* and soil and freshwater bacteria with similar phenotypic traits to that of the genus *Cytophaga*, but incapable of cellulose degradation were placed into the genus *Flexibacter* (Bernardet *et al.*, 1996). Several other species previously belonging to the genus *Flavobacterium* were also reclassified to new or different genera, including the genera *Bergeyella*, *Cytophaga*, *Empedobacter*, *Sphingobacterium*, *Weeksella*, *Myroides* and *Chryseobacterium* (Bernardet *et al.*, 1996; and Vancanneyt *et al.*, 1994). Bacterial cells belonging to the family *Flavobacteriaceae* are short to moderately long rods with parallel or slightly irregular sides and rounded or slightly tapered ends. They are usually 0.3 μm to 0.6 μm wide and 1 to 10 μm long although cell morphology may vary among members of the same species (Bernardet *et al.*, 2002). Flavobacterial cells may be non-motile or motile by gliding, which varies among genera. Gliding motility is an important characteristic for differentiating between genera classified in the family and between species of some of the genera. Colony pigmentation varies among genera from non-pigmented to pigmented by carotenoid or flexirubin pigments or both (Bernardet and Nakagawa, 2005). Carotenoid pigments are usually produced by members of marine species while flexirubin pigments are more frequently associated with clinical, freshwater or soil organisms (Reichenbach, 1989).

The genus *Flavobacterium* now forms the type genus of the family *Flavobacteriaceae*, and includes genera from diverse ecological niches with various physiological characteristics (Bernardet *et al.*, 2002). Habitats within which genera of the family *Flavobacteriaceae* are found are quite diverse and include soil; freshwater environments; marine environments; food and dairy products and their processing environments; clinical environments (humans, hospital equipment and medical devices); diseased dogs, cats, amphibians, and reptiles; diseased freshwater and marine fish, diseased mollusks, crustaceans, and sea urchins; diseased birds and eggs; digestive tract of insects; amoebae; and diseased plants (Bernardet and Nakagawa, 2005). Members of the CFB phylum usually constitute one of the major groups of heterotrophic bacteria of bacterial communities (Kirchman, 2002; O'Sullivan *et al.*, 2002; Brümmer *et al.*, 2000; Manz *et al.*, 1999; and Burchard and Sorogon, 1998). These organisms frequently participate in biofilms and seem to play an important role in biogeochemical cycles, through the production of diverse enzymes to degrade a variety of complex organic substrates (Johansen and Binnerup, 2002; and Kirchman, 2002). Members of the family *Flavobacteriaceae* have specifically been identified as part of bacterial communities in various environments (Bernardet and Nakagawa, 2005).

Members of the family *Flavobacteriaceae* produce various enzymes which enable them to degrade complex organic macromolecules including cellulose, pectin, xylan, chitin and agars. Proteolytic enzymes produced by these organisms play a role in the virulence of some of the pathogenic members of the family (Bernardet and Nakagawa, 2005). Fish-pathogenic *Flavobacterium* spp. produce proteases, which degrade collagen, elastin, fibrinogen and keratin, as well as polysaccharides of connective tissue, and are responsible for the characteristic lesions observed in flavobacterial fish diseases (Bernardet and Nakagawa, 2005).

Members of the family *Flavobacteriaceae* cause disease in humans, birds, fish and various other animals (Jooste and Hugo, 1999). Most members display opportunistic pathogenicity by invading immunocompromised hosts, and are often part of the normal host microflora or surrounding environment. This is the case for the potentially pathogenic members of the genera *Flavobacterium*, *Chryseobacterium*, *Myroides*, *Empedobacter*, *Bergeyella* and *Weeksella*. They have been implicated in serious

aquacultural diseases and thus, economic losses (Bernardet, 1998). In addition to BCWD, *F. psychrophilum* has been found to be the causative agent of rainbow trout fry syndrome (RTFS) in salmonid fish and occasionally other fish species, such as cyprinids and eel (Cepeda *et al.*, 2004; and Madetoja *et al.*, 2003). *F. columnare* is the causative agent of columnaris disease and has been denoted as one of the most important bacterial pathogens in freshwater fish (Tryanto and Wakabayashi, 1999). *Chryseobacterium scophthalmum*, formerly *F. scophthalmum*, was found to be present in coastal water samples in Scotland, and was isolated from wild turbot and found to be responsible for causing gill hyperplasia and haemorrhagic septicaemia (Bernardet, 1998).

2.1.1 The genera *Chryseobacterium* and *Elizabethkingia*

2.1.1.1 Taxonomy

Following extensive phylogenetic investigations, the genus *Flavobacterium* was emended, which gave rise to new genera including the genus *Chryseobacterium* (Bernardet *et al.*, 1996; and Vandamme *et al.*, 1994). The genus *Chryseobacterium* currently comprises 18 species namely, *C. balustinum* (Harrison, 1929), *C. indoltheticum* (Campbell and Williams, 1951), *C. indologenes* (Yabuuchi *et al.*, 1983), *C. gleum* (Holmes *et al.*, 1984), *C. scophthalmum* (Mudarris *et al.*, 1994), *C. 'proteolyticum'* (Yamaguchi and Yokoe, 2000), *C. defluvii* (Kämpfer *et al.*, 2003), *C. joostei* (Hugo *et al.*, 2003), *C. formosense* (Young *et al.*, 2005), *C. daecheongense* (Kim *et al.*, 2005a), *C. taichungense* (Shen *et al.*, 2005), *C. vrystaatense* (de Beer *et al.*, 2005), *C. shingense* (Shimomura *et al.*, 2005), *C. hispanicum* (Gallego *et al.*, 2006), *C. piscium* (de Beer *et al.*, 2006), *C. soldanellicola*, *C. taeanense* (Park *et al.*, 2006) and *C. wanjuense* (Weon *et al.*, 2006). Recently, the genus *Chryseobacterium* was divided, giving rise to a second genus, *Elizabethkingia* (Kim *et al.*, 2005b). Two former *Chryseobacterium* spp., *C. meningosepticum* and *C. miricola*, were allocated to this genus under the epithets *E. meningoseptica* and *E. miricola* (Kim *et al.*, 2005b). The division of the genus *Chryseobacterium* is a consequence of high-quality 16S rRNA gene sequencing which allows for better discrimination than the conservative DNA-rRNA hybridization

techniques, which were previously used for phylogenetic investigations of the genus *Flavobacterium*.

2.1.1.2 *Habitat*

Members of the genera *Chryseobacterium* and *Elizabethkingia* are found in diverse ecological niches, including clinical settings and various food products (Jooste and Hugo, 1999).

2.1.1.2.1 *Environmental habitats*

Chryseobacterium spp. are primarily isolated from soil, freshwater and marine environments. *C. indologenes* and *C. gleum* were isolated from soil and water by Tatum *et al.* (1974), a decade before they were described by Yabuuchi *et al.* (1983) and Holmes *et al.* (1984), respectively. Recently, *C. indologenes* isolates were isolated from soil samples in Indonesia and Spain, and showed great economical and environmental importance because of their ability to degrade toxic compounds (Lopez *et al.*, 2004; and Radianingtyas *et al.*, 2003). *C. indoltheticum* was isolated from marine mud samples (Campbell and Williams, 1951). '*C. proteolyticum*' was isolated by Yamaguchi and Yokoe (2000) from soil in a Japanese rice field. *C. daecheongense* from freshwater lake sediment (Kim *et al.*, 2005a), *C. taichungense* from contaminated soil (Shen *et al.*, 2005), *C. formosense* from the rhizosphere of garden lettuce (*Lactuca sativa* L.) and *C. wanjuense* was isolated from the surrounding soil (Weon *et al.*, 2006; and Young *et al.*, 2005), respectively. Two new species, *C. soldanellicola* and *C. taeanense* were isolated from roots of the sand-dune plants *Calystegia soldanella* (beach morning glory) and *Elymus mollis* (wild rye), respectively (Park *et al.*, 2006). *C. indologenes* strains were found among the very diverse bacterial community isolated from penguin guano collected from Antarctica (Zdonowski *et al.*, 2004). *E. meningoseptica* spp. are most often isolated from clinical settings, but have also been found to occur in soil and aquatic environments (Bruun and Ursing, 1987; Owen and Holmes, 1981; and Bruun, 1982).

Chryseobacterium spp. isolates are also prevalent in industrial environments. *C. defluvii* was isolated from activated sludge (Kämpfer *et al.*, 2003), and other

Chryseobacterium spp. were isolated from paper mill slimes (Oppong *et al.*, 2003). *C. hispanicum* was recently isolated from a drinking water distribution system by Gallego *et al.* (2006). *E. meningoseptica* was isolated from spent nuclear fuel pools (Bernardet *et al.*, 2006), and *E. miricola* from water condensation on the Russian space laboratory Mir (Li *et al.*, 2003).

Chryseobacterium and *Elizabethkingia* spp. exhibit varying degrees of tolerance to NaCl and environmental strains may occur both in freshwater and seawater, as well as in freshwater and marine fish (Bernardet *et al.*, 2006). There have been many reports of fish-associated members of the genera *Chryseobacterium* and *Elizabethkingia*. They have been found to be pathogenic or spoilage organisms and some belong to the normal fish microflora colonizing the mucus at the surface of the skin, gills and intestines of healthy fish, such as eel (Lijnen *et al.*, 2000) and salmon (Morita *et al.*, 1997).

There have been increasing reports of the isolation of *Chryseobacterium* and *Elizabethkingia* spp. from environmental biofilm communities. Four *Chryseobacterium* spp. were recovered from biofilm microflora in domestic sink drains (McBain *et al.*, 2003). Several *Chryseobacterium* spp. were among the common potentially pathogenic bacteria, which were isolated from samples of treated and untreated drinking water in South Africa (Pavlov *et al.*, 2004). *E. meningoseptica* strains were isolated from a slimy brown deposit inside the spouts of sink taps of a hospital during a disease outbreak (Hoque *et al.*, 2001).

2.1.1.2.2 Food environments

Chryseobacterium and *Elizabethkingia* spp. have been isolated from a variety of food products, and are often considered food spoilage organisms. They have been isolated from milk and dairy products, canned food products, poultry and poultry plants, meat and meat products, fish and fish products and vegetables during commercial processing (de Beer *et al.*, 2006; de Beer *et al.*, 2005; Young *et al.*, 2005; Hugo *et al.*, 2003; Gonzáles *et al.*, 2000; Austin and Austin, 1999; Jooste *et al.*, 1986b; and Owen and Holmes, 1981). The potential of these organisms to spoil milk and dairy products has been ascribed to their proteolytic and lipolytic activities (Cousin, 1982; and Gilmour and

Rowe, 1981). *C. balustinum*, *C. indologenes* and *C. gleum* have been isolated from milk products and implicated in the spoilage of these products (Jooste *et al.*, 1986). New genomic groups such as *C. joostei* have also been isolated from the dairy environment (Hugo *et al.*, 2003). *C. indologenes* and *C. gleum* have been isolated from meat and meat products (Owen and Holmes, 1981), and *C. vrystaatense* was isolated from raw chicken in a chicken-processing plant (de Beer *et al.*, 2005). *C. balustinum* was initially isolated from the scales of halibut in the Pacific Ocean (Brisou *et al.*, 1959; and Harrison, 1929), and was considered to be a fish product spoilage agent rather than a fish pathogen (Austin and Austin, 1999). Later, these organisms were also isolated from the skin and muscle of wild and farmed fish and were considered fish product spoilage organisms, since they were isolated from the fish stored for three days in melting ice (González *et al.*, 2000). The *C. balustinum*, *C. gleum* and *C. indologenes* strains isolated from Cape marine fish displayed various proteolytic activities, and thus may be involved in the proteolytic spoilage of fish products during early chill storage (Bernardet *et al.*, 2006).

2.1.1.2.3 *Human clinical and veterinary environments*

Among the genera *Chryseobacterium* and *Elizabethkingia*, *E. meningoseptica* is the species most commonly reported as a human pathogen. It constitutes a major clinical concern, since it is usually resistant to antibiotics used for the treatment of Gram-negative bacterial infections, including extended-spectrum β -lactams and aminoglycosides. Initially described as the causative agent for meningitis of premature neonatal patients (King, 1959), it is now together with other *Chryseobacterium* spp. isolates, associated with many other infections in immunocompromised and postoperative patients. *E. meningoseptica* has been found to cause endocarditis, cellulitis, abdominal infection, septic arthritis and eye infections in severely immunocompromised patients suffering from malignancy, end-stage hepatic and renal disease, extensive burns and acquired immune deficiency syndrome (AIDS) (Bernardet *et al.*, 2006). In a recent case report by Lee *et al.* (2006), the first cases of community-acquired necrotizing fasciitis and bacteremia caused by *E. meningoseptica* were reported. Lee *et al.* (2006) reported cases of *E. meningoseptica* infection without underlying infection, and questioned the

nosocomial opportunistic pathogenicity of this organism in immunocompromised hosts. *C. indologenes* has been associated with bacteremia, where the infection was related to indwelling devices such as central venous catheters and endotracheal tubes (Bernardet *et al.*, 2006). Urinary tract infection, ventilator-associated pneumonia, pyomyositis and infection of burn wounds by *C. indologenes* has also been reported (Hsueh *et al.*, 1997; and Hsueh *et al.*, 1996). *C. gleum* has also been associated with nosocomial infections in neonates and immunocompromised patients (Hoque *et al.*, 2001).

Although the pathogenicity of *Chryseobacterium* and *Elizabethkingia* spp. is not well described, these organisms have been isolated from diseased animal species. *C. indologenes* was isolated from diseased frogs (Olson *et al.*, 1992), *E. meningoseptica* from diseased frogs, turtles, birds, cats and dogs (Mauel *et al.*, 2002; Vancanneyt *et al.*, 1994; Jacobson *et al.*, 1989; Bruun and Ursing, 1987; and Sims, 1974), and *C. balustinum* and *C. scophthalmum* from diseased fish (Mudarris *et al.*, 1994). *C. balustinum* was isolated from the heart blood of a freshwater fish suffering from hemorrhagic septicemia, and *C. scophthalmum* has been found to cause gill hyperplasia and hemorrhagic septicemia in turbot species (Bernardet *et al.*, 2006). *Chryseobacterium* spp. have been found colonizing healthy eel, skin and muscle from wild and farmed freshwater fish, and intestines of salmon (*Oncorhynchus keta*), but were not implicated in causing disease in these fish during the investigations (Lijnen *et al.*, 2000; and Morita *et al.*, 1997). They are often thought of as fish product spoilage organisms rather than fish pathogens (Bernardet *et al.*, 2006). In contrast to González *et al.* (2000), Gennari and Cozzolino (1989) isolated *Chryseobacterium* spp. from fresh Mediterranean sardines (*Sardina pilchardus*) and found that the bacterial numbers decreased as the time in cold storage increased. The current view on *C. balustinum* pathogenicity is that it may occur in freshwater and seawater, and when present on the surface of fish it may be saprophytic, become an opportunistic pathogen or spoil the fish product during storage (Bernardet *et al.*, 2006). Recently, a new *Chryseobacterium* spp., *C. piscium* was isolated from fresh South Atlantic Ocean fish samples, and is considered to be involved in fish spoilage (de Beer *et al.*, 2006). *E. meningoseptica* has been isolated from diseased farmed koi carp suffering from skin lesions and hemorrhagic septicemia (Bernardet *et al.*, 2006).

2.1.2 The genera *Myroides* and *Empedobacter*

2.1.2.1 Taxonomy

The genera *Myroides* and *Empedobacter* also originated from the emendation of the genus *Flavobacterium* (Vandamme *et al.*, 1994). Previously known as *F. breve*, this organism was reclassified to the revived genus *Empedobacter* under the epithet *Empedobacter brevis* (Vandamme *et al.*, 1994). The first *Myroides* spp. were isolated in 1923 and classified as *Bacterium faecale aromaticum*, but were reclassified to *Flavobacterium odoratum* (Hugo *et al.*, 2005). Following extensive polyphasic and taxonomic studies, Vancanneyt *et al.* (1996) reclassified *F. odoratum* into the new genus *Myroides*. Currently, there are three described *Myroides* spp., *M. odoratus*, *M. odoratimimus* (Vancanneyt *et al.*, 1996) and *M. pelagicus* (Yoon *et al.*, 2006).

2.1.2.2 Habitats

E. brevis was originally isolated from canal water, but is mainly known to have a clinical origin (Bernardet *et al.*, 2006). *Empedobacter* spp. strains have been isolated from human eyes, bronchial secretions, peritoneal fluid, dialysis fluid, serous cavity fluid, cervixes and vaginas, wounds, blood and urine (Bruun, 1982; and Holmes *et al.*, 1978). The first *E. brevis* was pathogenic to guinea pigs, mice and rabbits (Holmes *et al.*, 1978). More recent clinical isolates have not been pathogenic, but may cause keratitis of the eyes when present with other bacterial species (Bottone *et al.*, 1992).

Myroides spp. have been isolated from various human specimens such as the intestine, urine, faeces, wound discharge, sputum and blood (Schreckenberger, 1998; and Hugo *et al.*, 2005). Although primarily isolated from clinical settings the pathogenic role of these organisms is still debated and they have been implicated in causing necrotizing fasciitis and bacteremia (Hugo *et al.*, 2005). Holmes *et al.* (1979) isolated *M. odoratus* from soft tissue in amputation sites and urinary tract infections. In 2000, *M. odoratimimus* was responsible for a major outbreak of urinary tract infection in Turkey (Yagci *et al.*, 2000). They have also been found to be widely distributed in the environment, mainly from aquatic sources (Hugo *et al.*, 2005). Engelbrecht *et al.* (1996) isolated *Myroides* spp. from South Atlantic fish species and from freshwater fish skin and

in the air during chill storage of freshwater fish (González *et al.*, 2000), where they caused spoilage of fish products. *M. pelagicus* was recently isolated from a consortium of crude oil-utilizing and – emulsifying bacteria from seawater (Maneerat *et al.*, 2005). *M. odoratus* has also been isolated from mixed-species biofilm communities developed on the surfaces of seafood processing plant equipment (Bremer *et al.*, 2001; and Tide *et al.*, 1999).

3. Biofilms – complex microbial communities

From the beginning of microbiology, scientists have studied microorganisms as planktonic, pure cultures. In reality, microorganisms have a strong tendency to colonize surfaces and thus, with the exception of a few rare cases, there are no free-swimming bacteria in nature. They are naturally found as sessile, highly structured multi-species communities, which are referred to as biofilms (Rickard *et al.*, 2003a).

A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002). Depending on the environment in which the biofilm has developed, non-cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components may also be enclosed in the biofilm matrix (Donlan, 2002). Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic environments (Donlan, 2002).

Biofilm communities are regarded as interactive organisms capable of significant collective activity (Shapiro, 1998; Shapiro and Dworkin, 1997; and Shapiro, 1988) or “microorganisms in a biofilm act together like one multicellular organism” (Netting, 2001). Initially, most of the research conducted on biofilms focused on pure bacterial cultures, which are rarely found in the natural environment, yet very important in disease. Increasing numbers of studies now focus on mixed-culture biofilm communities (Berry *et al.*, 2006; Hu *et al.*, 2003; Leonard *et al.*, 2000; Tide *et al.*, 1999; Elvers *et al.*, 1998; Leff *et al.*, 1998; and Jeong and Frank, 1994), as well as natural or environmental biofilms. Elvers *et al.* (1998) found that interactions such as neutralism, mutualism, commensalisms, amensalism, prey-predator relationships and competition may take place

in these complex, multispecies communities. Since biofilms are most often mixed consortiums of either bacteria or involving the microorganism kingdom, mixed biofilm community studies are of the essence. Regardless of how biofilm studies are conducted the most important practical reason for studying these communities is to find a way to control them.

3.1 Biofilm Architecture

The term 'biofilm' refers to any microbial organization which can range from patchy monolayers on some surfaces, very thick gelatinous masses associated with water cooling systems, to filamentous accretions near sewage outlets (Wimpenny *et al.*, 2000). Biofilm architecture is heterogeneous both in space and time, constantly changing because of external and internal processes (Donlan, 2002). In addition, biofilm architecture is dependent on the flow-rate in the surrounding environment and the composition of the biofilm (Poulsen, 1999). In heterogenic biofilms the architecture is often irregular due to the different growth and adherence patterns of the microorganisms (Costerton *et al.*, 1995). Although some structural attributes can generally be considered universal, every microbial biofilm community is in essence unique (Tolker-Nielsen and Molin, 2000).

At least three different structural organization views or models have been described for biofilm architecture (Wimpenny *et al.*, 2000). The traditional, planar homogenous view of biofilm structure originated from early studies on oral biofilms (Nyvad and Fejerskov, 1997). The 'heterogeneous mosaic' model was described from examination of the inner surfaces of water distribution systems (Keevil and Walker, 1992). These biofilms consisted of microcolonies held together by extracellular polymeric substances (EPS) and appeared as columns surrounded by a liquid phase in which grazing protozoa were observed (Keevil and Walker, 1992). Below the microcolonies, a thick, homogeneous layer of cells attached to the substratum was observed (Keevil and Walker, 1992). The 'mushroom or tulip' model is currently the most described and accepted model for biofilm architecture (Wimpenny *et al.*, 2000). In this model the mature biofilm structure consists of intricate architecture featuring

columns, interstitial voids and typically, though not necessarily, mushroom-like towers with stalks narrower than the upper surface parts (Netting, 2001). Within this elaborate architecture, niches are formed throughout the spatially well-organized system, which provides an opportunity for metabolic co-operation. This allows these organisms to exist in a mutualistic multi-species consortium (Davey and O'Toole, 2000). The biofilm has a porous structure with capillary water channels within which water and nutrients are distributed (Costerton *et al.*, 1995). These water channels are found both under and between the biofilm microcolonies and are believed to also participate in the transport of oxygen to the inner areas of the biofilm (Poulsen, 1999). Transport of nutrients to the bottom of the biofilm occurs through special capillary tubes. Cellular waste is also secreted through capillary tubes to the surface of the biofilm (Costerton *et al.*, 1995).

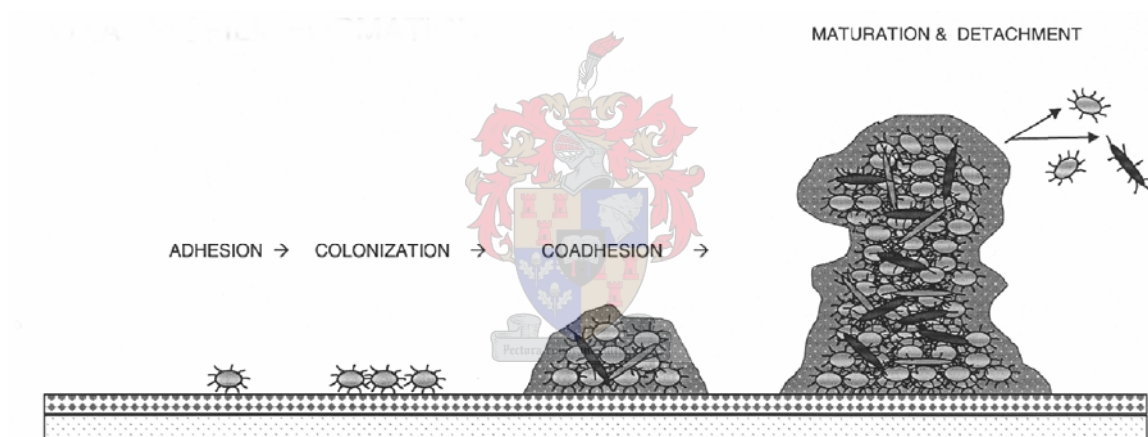


Figure 1. 1 Diagram illustrating the sequence of development of a mature biofilm structure (Schele and Petersen, 2004).

3.2 Extracellular Polymeric Substances

EPS has been considered the primary matrix material of biofilms, and may account for up to 90% of the total organic carbon of biofilms (Flemming *et al.*, 2000). EPS is primarily composed of polysaccharides, but may vary in chemical and physical properties (Sutherland, 2001). Anionic properties of the EPS are due to the presence of uronic acids or ketal-linked pyruvates (Sutherland, 2001). The anionic property plays an important role as it allows for the association of divalent cations such as calcium and

magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Flemming *et al.*, 2000). Large amounts of water is also incorporated in the EPS structure through hydrogen bonding (Sutherland, 2001). Two important properties of EPS that may have an effect on the biofilm include the composition and structure of the polysaccharides, which determines the primary conformation of the biofilm, and spatial and temporal variation in EPS composition, which determines the architecture of the mature biofilm (Sutherland, 2001). EPS production is known to be affected by the nutrient concentration of the growth medium, where EPS synthesis is promoted in conditions where carbon is available in excess and nitrogen, potassium, or phosphate is limited (Sutherland, 2001). The presence of EPS surrounding biofilm communities is frequently correlated with adhesion and protection of the cells contained in the biofilm (Costerton *et al.*, 1995; Vess *et al.*, 1993; and Raad *et al.*, 1991). Because EPS is highly hydrated, it prevents desiccation in some biofilms, and may also contribute to the antimicrobial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm (Donlan, 2000).

3.3 Process of Biofilm Formation

Three methods for biofilm formation have been described (Stoodley *et al.*, 2002). The first method is the relocation of cells attached to a substrate through surface translocation (Dalton *et al.*, 1996; and Korber *et al.*, 1995). The type IV pili allows for such relocation of *P. aeruginosa* cells already attached to surfaces (O'Toole and Kolter, 1998). Furthermore, binary division of attached cells also results in the formation of biofilms as the daughter cells produced spread horizontally and vertically to form microcolonies (Heydorn *et al.*, 2000). Finally, coaggregation between attached cells and free-swimming cells from the surrounding bulk fluid also allow for development of the biofilm (Tolker-Nielson *et al.*, 2000). The morphology of a mature biofilm community varies depending on environmental conditions, such as the location, nature of the microbes and nutrient availability. They may be thick multi-layered lawns of cells such as those found in dental plaque biofilms, dispersed microcolonies or thin layers of cells with protrusions (Wimpenny and Colasanti, 1997).

There are various positioning mechanisms, which facilitate the initiation of a biofilm structure. These mechanisms include flagellar motility, which has been described as the most common. Other mechanisms include the various methods of surface translocation such as twitching, gliding, darting and sliding (Henrichsen, 1972). Modulation of density or regulation of buoyancy by *Amoebobacter purpureus* is also considered a positioning mechanism (Overmann and Pfennig, 1992). Magnetosomes, which cause certain bacterial cells to align with the earth's geomagnetic field and limit their lateral movement, have also been implicated in bacterial cell positioning (Schuler and Frankel, 1999; and Bazylinski, 1995). The most important positioning mechanisms are considered aggregation or attachment. The various factors that may influence attachment and biofilm formation are summarized in Table 1.1.

Table 1.1 Variable factors important in cell attachment and biofilm formation (Wimpenny *et al.*, 2000).

Properties of the substratum	Properties of the bulk fluid	Properties of the cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Fimbriae
Conditioning film	Temperature	Flagella
	Cations	Extracellular polymeric substances
	Presence of antimicrobial agents	

3.3.1 Attachment

There is still controversy with regards to whether initial attachment does indeed influence subsequent biofilm formation. Some scientists have found that biofilm formation was not dependent on this initial attachment event (Cerca *et al.*, 2005; and Chae *et al.*, 2005). Others have shown that biofilm formation is preceded by initial attachment to a solid substrate, which involves various specific and non-specific physico-chemical interactions (Rickard *et al.*, 2003a). Various mechanisms of attachment have been described and these mechanisms utilize different cell components. Outer membrane proteins (OMPs); wall polysaccharides such as capsules, lipopolysaccharides (LPS); and cell surface agglutinins are examples of such components (Davey and O'Toole, 2000). Makin and Beveridge (1996) demonstrated that loss of the B-band LPS reduced the

ability of *P. aeruginosa* cells to interact with hydrophilic surfaces. Danese *et al.* (2000) showed that the major phase-variable OMPs in *Escherichia coli*, Ag43, was directly required for the cells' interaction with a surface (Danese *et al.*, 2000). The precise process of initial attachment involves three steps: adsorption, adhesion and adherence.

3.3.1.1 Adsorption

Adsorption involves physical chemistry, which includes attraction by ionic forces, and loose binding interactions such as van der Waals' forces. Adhesion is a reversible stage, and involves recognition of surface sites or adhesins, which could include either proper anchorage devices with precise structures, or could be gels, mucuses or soluble substances. During this stage the bacteria exhibit several species-specific behaviours, which include rolling, creeping and aggregate formation before they begin to produce small amounts of exopolysaccharide and move on to the next stage (Korber *et al.*, 1995).

3.3.1.2 Adhesion

Adherence is the irreversible binding of the organism to a surface through specific adhesins. Lectins are a heterogeneous group of glycoproteins, which can be found in all organisms from viruses and bacteria to mammals. They act as recognition, adhesion or signaling molecules (Hansen and Olafsen, 1999). In invertebrates, lectins play a role in defense against microorganisms by acting as antibodies that attach to invading microbes to make them more susceptible to phagocytosis (Hansen and Olafsen, 1999). In mammals, there are various lectin-like structures which play a role in immunity (Kéry, 1991). In *E. coli*, the flagella and type I pili (mannose-sensitive adhesins) were found to be responsible for adhesion to polyvinyl chloride (PVC). The flagellum is used to overcome repulsive forces, which act on the microorganisms from abiotic surfaces. When the cell reaches the surface, type I pili are responsible for stable cell-to-surface adherence. Once attached, cell motility allows for the movement of the cell along the surface to reach desirable areas (Pratt and Kolter, 1998). O'Toole and Kolter (1998) found that in *P. aeruginosa* biofilm formation, flagellar movement is also necessary to propel the cell toward the surface. In this case type IV pili (encoded by *pilA*) are

responsible for twitching motility, which moves the cell along the adhered surface. Flagella are important for attachment and formation of initial biofilm monolayers, while pili play a part in the production of confluent films and the accumulation of cells in microcolonies (O'Toole and Kolter, 1998). Another study also implicated pili in biofilm formation of *Salmonella enteritidis* to stainless steel and Teflon (Austin *et al.*, 1998). Thin aggregative fimbriae SEF17, were found to be responsible for cell-to-cell contact during biofilm formation (Austin *et al.*, 1998). Although cell structures such as flagella, pili and other surface proteins have been found to play a role in biofilm formation, one cannot assign a specific task to each structure, since the various structures play different roles in this process, in different microbial species (Stickler, 1999).

3.3.1.3 *Adherence*

Attachment is mediated by various effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium and cell surface properties (Donlan, 2002).

3.3.1.3.1 *Substratum effects*

Surface roughness has been found to increase microbial attachment to surfaces. This is due to a decrease in shear forces and an increase in surface area (Characklis *et al.*, 1990). Physicochemical properties of the surface have also been found to influence attachment of microbial cells to surfaces. Certain hydrophobic interactions that occur between the cell surface and the substrate enable the cell to overcome repulsive forces and attach to the surface. Most investigators have found that microorganisms attach more rapidly to hydrophobic, non-polar surfaces such as plastics than to hydrophilic materials such as glass or metals (Bendinger *et al.*, 1993; Pringle and Fletcher, 1983; and Fletcher and Loeb, 1979)

3.3.1.3.2 *Conditioning films*

A surface exposed in an aqueous medium will become coated by polymers from the medium to form a conditioning film, which could affect the rate of microbial cell

attachment (Loeb and Neihof, 1975). An example of such a film is the proteinaceous acquired pellicle, which develops on tooth enamel preceding biofilm formation in the oral cavity. Marsh (1995) investigated the composition of the pellicle and found that it contained albumin, lysozyme, glycoproteins, phosphoproteins, lipids and gingival crevice fluid. Attachment of bacteria to biomaterials was increased by host-produced conditioning films such as blood, tears, urine, saliva, intravascular fluid and respiratory secretions (Mittelman, 1996). The adherence of *S. putrefaciens* and *S. enterica* serovar Typhimurium to stainless steel was found to be increased by the presence of a conditioning film (Bagge *et al.*, 2001; and Hood and Zottola, 1997). In contrast, Barnes *et al.* (1999) found a decrease in attachment of *P. fragi* and *Listeria monocytogenes* when an organic film was present. Certain food components such as milk proteins were found to decrease bacterial attachment (Barnes *et al.*, 1999).

3.3.1.3.3 *Hydrodynamics of the aqueous medium*

The influence on biofilm formation under increased shear forces when placed under high flow rates of the aqueous medium has also been described. Under these conditions, biofilm cell clusters became elongated in the downstream direction of unidirectional flows to form filamentous streamers (Stoodley *et al.*, 1999). This has been documented for *P. aeruginosa* biofilms where the streamers were attached to a substrate by an upstream “head” portion and a downstream “tail” portion that freely oscillated in the flow. They also showed that the streamers become elongated over time, with the tails getting thinner until there was only a small chain of single cells at their tips (Stoodley *et al.*, 1998). High shear forces also influence the physical properties of biofilms such as density and strength. Liu and Tay (2001) showed that biofilms grown under high fluid shear were smoother and denser than those grown under low shear forces. *Desulfovibrio* spp. and *P. aeruginosa* biofilms were found to be more rigid and stronger when grown under high shear forces (Stoodley *et al.*, 2001). Fluid flow velocity (and associated shear rates) has been suggested to be important in the development of biofilm community structure (Beyenal and Lewandowski, 2002) and govern the abilities of individual species to immigrate to biofilms and to colonize new surfaces (Cloete *et al.*, 2003; and Stoodley

et al., 1999). The effect of shear rate on bacterial composition and diversity of freshwater biofilms has not been studied extensively, but evidence is emerging that high fluid velocities with associated high shear forces lead to the development of less diverse biofilm communities as compared to those developed at lower shear rates (Rickard *et al.*, 2004b; Cloete *et al.*, 2003; Rickard *et al.*, 2003b; Liu and Tay, 2002; and Soini *et al.*, 2002).

3.3.1.3.4 *Characteristics of the medium*

The composition of growth medium has been shown to affect biofilm formation by microorganisms. According to McEldowney and Fletcher (1986), the nutrient level of cultivation media affected the adhesion and biofilm formation of some bacteria. Kim and Frank (1994) observed an increased attachment by *L. monocytogenes* in low-nutrient containing medium. *E. coli* O517:H7 is reported to form a biofilm only in low-nutrient media (Dewanti and Wong, 1995), while other bacterial strains, *E. coli* K-12 and *Vibrio cholerae*, will not form biofilms in minimal media (Watnick *et al.*, 1999; and Pratt and Kolter, 1998). It has also been observed that substrate concentration also plays a part in the dynamics of biofilm structure (Wimpenny *et al.*, 2000). This was first demonstrated in a study of anaerobic bacteria forming colonies in a continuous flow cell system with varying substrate concentrations, where colony size was directly correlated with the substrate concentration in the flowing media (Szewzyk and Schink, 1987). Mushroom-like structures observed in biofilm architecture have been found to develop in intermediate substrate concentrations, under continuous flow in laboratory systems (Wimpenny *et al.*, 2000).

3.3.1.3.5 *Cell surface properties*

Cell surface hydrophobicity, presence of fimbriae and flagella, and EPS production all influence the rate of attachment by microbial cells to surfaces (Donlan, 2002). Korber *et al.* (1989) demonstrated that motile cells attached more readily to surfaces than non-motile cells. Redistribution to vacant areas on surfaces, and colonization of these areas by non-motile bacteria is also not as successful as with motile

bacteria. Early studies have suggested that the overall hydrophobicity of a bacterium could serve as a good predictor of the surface that an organism might colonize (Costerton *et al.*, 1995).

The importance of cell-surface hydrophobicity in adherence of bacterial cells to surfaces is controversial. Some authors have shown that hydrophobic interactions contribute in the adherence process (Bos *et al.*, 1999; van Loosdrecht *et al.*, 1987; Paul and Jefferey, 1985; and Rutter and Vincent, 1980), while others have found no correlation between hydrophobicity and adherence (Cerca *et al.*, 2005; and Bandin *et al.*, 1989). Balebona *et al.* (2001) observed no correlation between adherence to host tissue and hydrophobicity for *Vibrio* spp. isolated from infected fish. Additionally, it has been proposed that whole cell hydrophobicity is responsible for enhancing autoaggregation interactions in biofilm communities (Kos *et al.*, 2003; Del Re *et al.*, 2000; and Ljungh *et al.*, 1985). Rickard *et al.* (2004a) showed that hydrophobicity, together with coaggregation and autoaggregation, are important cellular properties for colonization of surfaces and subsequent biofilm development.

3.3.2 Coaggregation

The first organisms to colonize an area suitable for multi-species biofilm communities are referred to as primary colonizers (Fig. 1.2a). In suitable conditions these primary adherers will multiply and cover the substratum (Fig. 1.2b), and secondary colonizers will then attach to the primary adherers (Fig. 1.2c) to develop a multi-species community (Fig. 1.2d) (Rickard *et al.*, 2003a). Maturation of a biofilm community relies on specific cell-to-cell recognition between two or more genetically distinct strains, referred to as coaggregation. Coaggregation plays an important role as an adhesion mechanism involved in integrating and establishing bacteria into a biofilm community (Rickard *et al.*, 2002a). There are two ways by which coaggregation may contribute to the development of a mature biofilm community (Fig. 1.2c). Firstly, single cells in the surrounding environment may recognize and adhere to genetically distinct cells already adhered to a surface. Secondly, secondary colonizers may coaggregate in suspension and

subsequently, this coaggregate then adheres to the developing biofilm (Rickard *et al.*, 2003a).

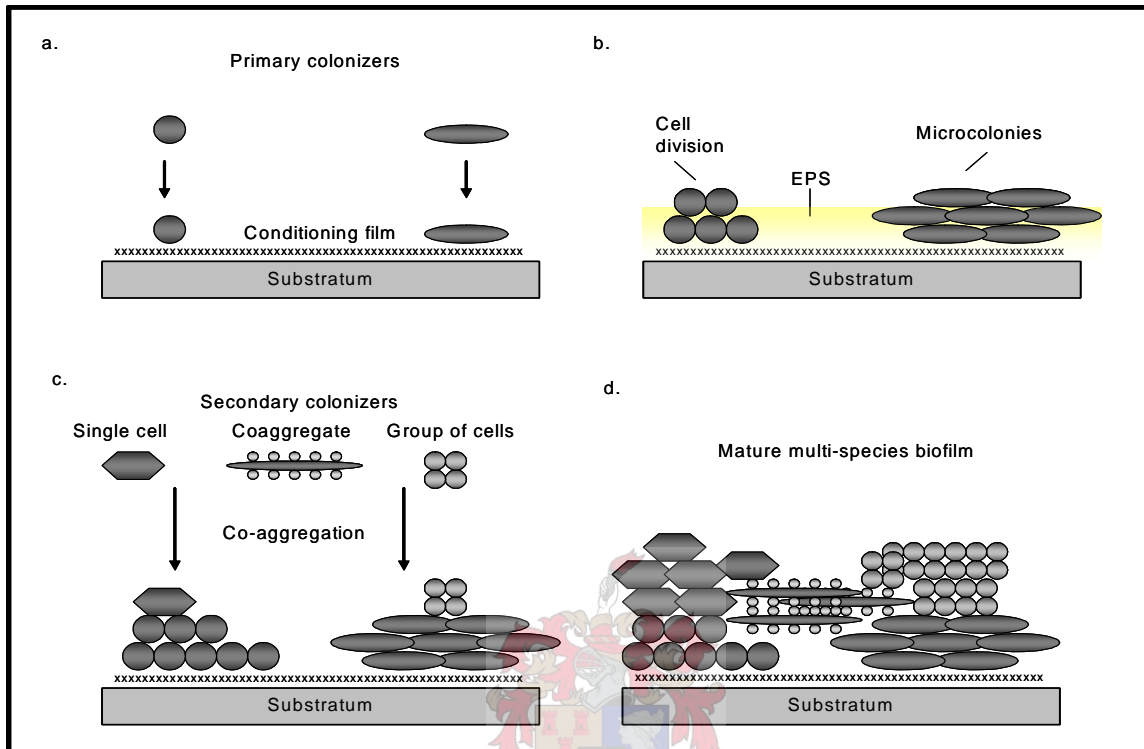


Figure 1.2 Diagram illustrating the role of coaggregation in the maturation of biofilm communities. (a) Colonization by primary colonizers of a substratum covered in a conditioning film; (b) Formation of microcolonies through cell division, cell growth and production of EPS; (c) Coaggregation of secondary colonizers; (d) Maturation of the multi-species biofilm. Adapted from (Rickard *et al.*, 2003a).

Coaggregation was first described by Gibbons and Nygaard (1970) between oral plaque bacteria, and may occur between organisms of the same or different genera. Initially, it was suggested that most strains did not coaggregate with members of their own genus, with the exception of extensive partnerships that had been documented for members of the genus *Actinomyces* and *Streptococcus* (Kolenbrander *et al.*, 1990). Intraspecies coaggregation has been suggested to be a phenomenon unique to freshwater biofilm bacteria (Rickard *et al.*, 2002a). The sequence of biofilm formation and maturation may be deduced through coaggregation studies. *Fusobacterium nucleatum*, found in dental plaque, is able to coaggregate with all other oral bacteria (Andersen *et al.*,

1998; and Kolenbrander *et al.*, 1995). Due to the fact that it is a non-selective coaggregator, it has been described as a bridging organism, which enables the linking between primary and secondary colonizers that are unable to coaggregate with each other (Kolenbrander *et al.*, 1999). Similarly, it has been reported that *Blastomonas natatoria* may play a similar role in aquatic biofilms (Rickard *et al.*, 2002b).

Studies on the coaggregation of oral bacteria have revealed that coaggregation is a highly specific process which is mediated by lectin-saccharide interactions between cell surface molecules on the partner organisms (Kolenbrander, 1997; Ebisu *et al.*, 1988; Kagermeier and London, 1986; and Bourgeau and McBride, 1976). Receptor partners for adhesins are believed to be cell-wall associated polysaccharides as identified in *S. sanguis* (Cassels and London, 1989) and *S. oralis* (McIntire *et al.*, 1988). Other bacterial structures or molecules involved in coaggregation of oral bacteria include protein adhesins on the surface of *Capnocytophaga gingivalis* and *F. nucleatum* (Kinder and Holt, 1989; Kolenbrander, 1989; and Kolenbrander, 1988), and the carbohydrate receptor on *S. sanguis* (Kolenbrander, 1989; and Kolenbrander, 1988). Coaggregation interactions have also been described for urogenital flora (Kmet and Lucchini, 1997; and Reid *et al.*, 1988), the intestinal tract of humans (Drago *et al.*, 1997) and pigs (Kmet *et al.*, 1995), between strains of *Lactobacillus* from chicken crops (Vandevoorde *et al.*, 1992), and aquatic biofilm-forming bacteria (Rickard *et al.*, 2002a; Rickard *et al.*, 2000; and Buswell *et al.*, 1997). Rickard *et al.* (2000) investigated surface-associated molecules involved in coaggregation between the two aquatic bacterial strains, *B. natatoria* and *Micrococcus luteus* by heat, protease treatment and sugar reversal tests. Coaggregation by these two aquatic strains was also found to be mediated by lectin-saccharide interactions (Rickard *et al.*, 2000). Taweechaisupapong and Doyle (2000) observed that even though bacteria may possess different kinds of adhesins, sensitivity to chelating agents is a common property among bacteria. They proposed that the reversal of coaggregation by chelating agents, such as carboxymethylcellulose and citrate, should be investigated further for its anti-adhesin properties in the control of biofilm communities (Taweechaisupapong and Doyle, 2000).

Autoaggregation was proposed to be a mechanism whereby a bacterial strain within a biofilm expressed polymers to enhance the integration of genetically identical

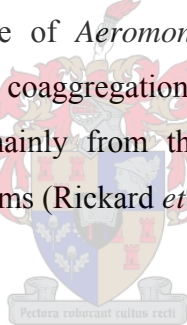
strains (Rickard *et al.*, 2003b). Autoaggregating strains of freshwater bacteria were found to be numerically dominant in freshwater biofilm communities, and autoaggregation was found together with coaggregation to enhance the development of freshwater biofilms (Rickard *et al.*, 2003b). Intermediate shear rates were observed to select for the highest proportion of coaggregating bacteria, whereas higher shear rates resulted in a higher proportion of autoaggregating bacteria (Rickard *et al.*, 2004b). Therefore, they proposed that coaggregation may only weakly enhance cell-cell attachment (as opposed to autoaggregation), but mediated juxtapositioning of species next to favourable partner species within taxonomically diverse biofilms (Rickard *et al.*, 2004a). At higher shear rates, biofilm diversity decreases and cell physiology changes as to select for autoaggregation to allow for genetically similar bacterial cells to withstand shear forces and remain within the colonized area (Rickard *et al.*, 2004b).

3.3.2.1 Coaggregation adhesins

Various cell-surface components mediating coaggregation have been identified in oral biofilm bacteria, the majority isolated are those found on spp. of *Streptococcus*, *Actinomyces* and *Fusobacterium* (Kolenbrander, 2000). *S. gordonii*, for example, carries five distinct proteins involved in coaggregation interactions (Rickard *et al.*, 2003a). One of these is a protein adhesin, which facilitates intrageneric, galactoside-inhibitable coaggregation with other streptococcal spp. (Clemans *et al.*, 1999). A lectin-like protein adhesin on the surface of *S. gordonii* has been associated with its ability to adhere to components of saliva, polymorphonuclear leucocytes and other spp. of dental plaque (Takahashi *et al.*, 2002; and Takahashi *et al.*, 1997). This organism also possesses thin, peritrichous fibrils, which contain the adhesin molecule for coaggregation with *A. naeslundii* (McNab *et al.*, 1999). It has been found that oral biofilm bacteria often carry adhesin molecules on surface structures projecting away from the cell, such as fimbriae and fibrils (Handley *et al.*, 2001). This helps the coaggregating partner organisms overcome the electrostatic barrier between them, and make effective contact with each other (Busscher *et al.*, 1992). Finally, a specific adhesin for the coaggregation with *Porphyromonas gingivalis* has also been identified on the surface of *S. gordonii* (Love *et*

al., 2000). Surface adhesins do not necessarily always recognize specific coaggregating partners. The bridging organism in dental plaque, *F. nucleatum*, has three multifunctional adhesin molecules that mediate the coaggregation with seven different genera of plaque bacteria as well as the adhesion to some host surfaces (Kolenbrander *et al.*, 1999; and Shaniztki *et al.*, 1998).

Coaggregation among freshwater bacteria is known to occur between more than 15 different bacterial genera. The adhesins are lectin-like proteins, and the receptors contain residues of galactose, galactosamine or lactose (Rickard *et al.*, 2003b; and Rickard *et al.*, 2002a). A sialic acid-binding lectin has been found to be involved in the adhesion abilities of the fish pathogen *F. psychrophilum* (Møller *et al.*, 2003). Carbohydrate-binding lectins have also been described for other aquaculture bacterial spp. including *F. columnare*, and members of the genera *Pseudomonas* and *Vibrio* (Møller *et al.*, 2003; and An and Friedman, 2000). In addition, protein adhesins have been found present on the surface of *Aeromonas* spp. (An and Friedman, 2000). Although not much is known about coaggregation receptors, it has been suggested that coaggregation specificity results mainly from the high diversity of unique adhesin molecules present on partner organisms (Rickard *et al.*, 2003b).



3.3.3 Quorum Sensing

A population density-dependent cell-cell signaling mechanism, or quorum sensing, regulates a range of biological functions in bacteria and these extracellular signaling molecules have been implicated in biofilm formation (Davies *et al.*, 1998). By monitoring the presence of these self-produced extracellular signaling molecules, bacterial cells are able to sense their population density. When the population density is significantly high, the accumulated signals trigger the expression of target genes to initiate a new set of biological activities (Zhang, 2003; and Parsek and Greenberg, 2000). In Gram-negative bacteria, quorum sensing is achieved through the activity of acylated homoserine lactones (AHLs) (Davies *et al.*, 1998).

AHLs have been detected from diverse environments including natural biofilms growing on submerged stones taken from a river and from biofilm communities on

urethral catheters (Stickler *et al.*, 1998; and McLean *et al.*, 1997). The AHL-dependent quorum sensing system has been found to control biofilm formation in *A. hydrophila*, *Burkholderia cepacia* and *Serratia liquefaciens* (Kjelleberg and Molin, 2002). Davies *et al.* (1998) showed that the development of biofilm structures is influenced by AHLs. *P. aeruginosa* AHL mutants produced thin layers of cells on a surface, but wild-type biofilm morphology was restored by the addition of the AHL. Additionally, accumulation of AHLs in developing biofilms was found to be responsible for the transformation of planktonic to sessile cells and coordination of their behaviour to form complex communities (Davies *et al.*, 1998). Hu *et al.* (2003) studied the production of AHL quorum sensing molecules, and found that *P. aeruginosa* and *Enterobacter agglomerans* produced these molecules. *Agrobacterium tumefaciens*, *Bacillus cereus* and *Ralstonia* spp. were found to produce AHL degradation enzymes, which may indicate a certain level of regulation in water reclamation system biofilms (Hu *et al.*, 2003).

Bruhn *et al.* (2005) investigated the production of quorum sensing AHL molecules among selected strains of Gram-negative fish bacterial pathogens. AHLs were produced by all the strains of *A. salmonicida*, *A. hydrophila*, *Y. ruckeri*, *V. salmonicida*, and *V. vulnificus* (Bruhn *et al.*, 2005). Additionally, AHL production was strain-specific for *V. splendidus* and negative for *F. psychrophilum* (Bruhn *et al.*, 2005). Although these quorum sensing molecules were detected in these fish pathogens, their role in the formation of aquatic biofilms has not yet been completely elucidated (Bruhn *et al.*, 2005). Cell-to-cell communication has also been found to play an important role in biofilm formation by *S. gordonii*, the primary colonizing organism in oral biofilm communities (Loo *et al.*, 2000). In contrast, Van Houdt *et al.* (2004) showed that the production of AHL molecules and other quorum sensing molecules did not play a significant role in the biofilm formation of Gram-negative bacteria isolated from a raw vegetable processing environment.

3.4 Advantages to microorganisms

Growth as part of multi-species communities confers many advantages to the microbial species in biofilms. One such advantage is that microbes in a biofilm are able to combine and thus enhance metabolic activities, which allows for the degradation and subsequent use of substrates, which planktonic cells would normally not be able to utilize (Rickard *et al.*, 2003a). Examples of these include: glutamate metabolism (Costerton *et al.*, 1987), and colonization of an area in close proximity to specific substrates, such as the adherence of cellulolytic bacteria to cellulose (Costerton *et al.*, 1987). *Campylobacter rectus* produces a protoheme which functions as a growth factor for *P. gingivalis*, and the growth of *C. rectus* is stimulated by formate produced by *Prevotella melaninogenica* (Grenier and Mayrand, 1986). This indicates the reliance of some organisms on other microbes for growth and, therefore, their dependence on mixed biofilm communities for survival. Furthermore, the presence of pathogenic organisms as part of biofilms in animal hosts, allows for better access and delivery of toxic substances to host tissue cells, which inevitably increases pathogenicity (Kinder and Holt, 1994).

EPS or biofilm matrix, which has been found surrounding the biofilm communities, also confers an advantage to the organisms in the biofilm. It acts as a barrier, prohibiting diffusion of nutrients, which have been derived from the metabolism of substrates by the microbial community, or collected from the surrounding environment, and thus maintaining high nutrient availability within the biofilm (Kinder and Holt, 1994). The biofilm matrix also limits the access of harmful substances and predators, and protects encased cells from environmental changes, such as shear forces, pH changes, oxygen radicals and disinfectants (Jefferson, 2004). EPS also protects biofilm microbes from the immune system in animal hosts by hiding antigens, which are then less noticed by the immune system (Kinder and Holt, 1994).

Cvitkovitch *et al.* (2003) observed that microbial biofilms are excellent environments for horizontal transfer of genetic material between microbial species. The minimal shear forces and close cell to cell contact in a biofilm, creates a perfect niche for this transfer to take place (Donlan, 2002). Competent bacteria in a biofilm can rapidly and easily take up plasmid DNA through conjugation with other microbes, and this environment also allows for the spread of phage DNA (Harisson *et al.*, 2005; and Donlan,

2002). Ghigo (2001) described conjugative F-plasmids in *E. coli* that encode pili, which adhere non-specifically to surfaces and other bacteria and thereby it facilitating its own transfer through horizontal gene transfer. Roberts *et al.* (1999) demonstrated horizontal gene transfer in a microcosm dental plaque where a conjugative transposon from *B. subtilis* was transferred to a *Streptococcus* biofilm. Assessment of the antibiotic resistance of the biofilm bacteria revealed tetracycline resistant *Streptococcus* spp., indicating transfer of the conjugative transposon. This study revealed not only gene transfer in oral microbial biofilms, but also transfer of genetic elements between oral and non-oral bacteria (Roberts *et al.*, 1999). It has also been demonstrated that organisms that usually only form microcolonies, were able to form biofilms when they received plasmids through conjugation by plasmid-carrying strains (Donlan, 2002).

Microorganisms in biofilm communities are able to withstand antibiotic doses as much as 10 to 1000 times more concentrated (as compared to their planktonic counterparts) (Lewis, 2001; Mah and O'Toole, 2001; Costerton *et al.*, 1999; and Nickel *et al.*, 1985). The mechanisms by which bacterial cells in biofilms are able to withstand antimicrobial agents are still widely disputed (Fig 1.3). Due to their close proximity, biofilm bacteria are able to communicate through intracellular communication. This allows for a change in physiology by these bacteria when antibiotics are threatening the community, and certain organisms are able to produce molecular pumps, which expel antibiotics (Harrison *et al.*, 2005). Similarly, Suci *et al.* (1998) suggested that due to the biofilms' protective nature, cells in the biofilm are able to undergo a physiologically protective set of changes before the antimicrobial agents are able to penetrate the protective layers and reach the cells. It was also proposed that cells in the bulk of the biofilm are not physiologically active, which could explain the reduced susceptibilities to antimicrobial agents (Huang *et al.*, 1998; and Xu *et al.*, 1998). Stewart *et al.* (1998) suggested that population density may be responsible for the increased resistance of *E. aerogenes* biofilms to disinfectant chlorine treatments. Certain cells that are present on the boundaries of a biofilm have been identified as persister cells. These bacteria have the ability to withstand antibiotic treatment by not growing in the presence of antibiotics, but amazingly do not die either. Therefore, persister cells block the entry of antibiotic substances, and once the antibiotics are no longer a threat these cells continue to grow

normally (Harrison *et al.*, 2005). EPS is thought to play a major role in protection of the biofilm organisms from antibiotic substances in many ways. Antibiotic-degrading enzymes, such as β -lactamases, produced by some organisms may be collected in the surrounding EPS. This leads to a higher concentration of these enzymes in one area, as opposed to the amount of enzyme produced by planktonic cells, and thus better degradation of β -lactam antibiotics (Harrison *et al.*, 2005). EPS also carries a negative charge, and is thus able to bind and prevent entry, and access to biofilm organisms, of positively-charged antibiotics (Harrison *et al.*, 2005) (Fig. 1.3).

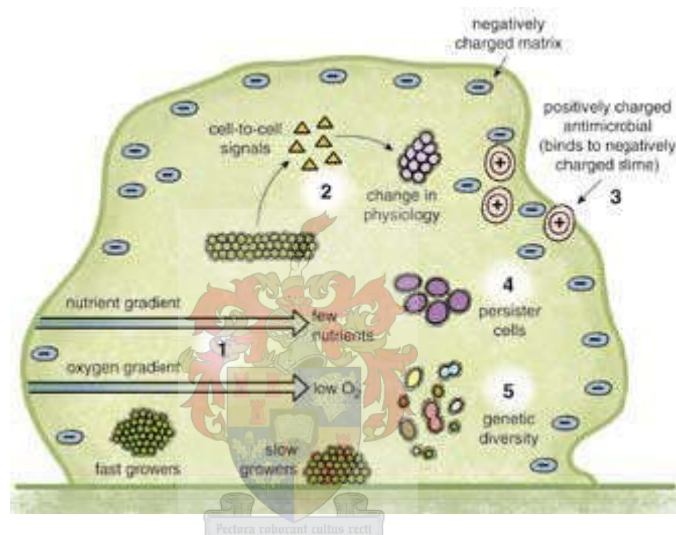


Figure 1.3 Diagram illustrating mechanisms of antimicrobial resistance by biofilm communities. (1) Bacteria near the centre of the microcolony grow slower because they are exposed lower concentrations of oxygen and nutrients and are thus spared the effects of antimicrobial substances. Intercellular signals (2) can alter the physiology of the biofilm causing members to produce molecular pumps that expel antibiotics from the cells and allow the community to grow even in the presence of a drug. The biofilm matrix is negatively charged (3) and so binds to positively antimicrobials, preventing them from reaching the cells within the colony. Persister cells (4) do not grow in the presence of an antibiotic, but neither do they die. Upon removal of the drug, the persisters can give rise to a normal bacterial colony. Finally, population diversity (5), genetic as well as physiological, improves the chance that some cells will survive adverse environmental conditions. (Harrison *et al.*, 2005)

3.5 Disadvantages to industries

Microbial biofilms have been found in various different environments, such as living tissues, medical devices, food processing plants, industrial or potable water system

piping or natural aquatic systems (Donlan, 2002). They have attracted a lot of interest in medical, industrial and environmental industries due to great concern for the potential harm they may cause.

3.5.1 Food-processing industry

In the food-processing industries (seafood, meat and dairy processing plants) biofilm formation has also been investigated extensively (Poulsen, 1999). Through attachment, surface growth and biofilm formation, bacteria are able to accumulate on food preparation surfaces (Van Houdt *et al.*, 2004; Donlan, 2002; and Poulsen, 1999). In addition, food-spoilage organisms that are not naturally inclined to form sessile communities may associate with biofilm-forming communities of food processing surfaces and become increasingly resistant to surface sanitizers (Leriche and Carpentier, 1995; and Sasahara and Zottala, 1993). Bremer *et al.* (2001) studied the survival of *L. monocytogenes* attached to stainless steel surfaces in a food processing environment. The attachment of *L. monocytogenes* to these surfaces increased significantly in the presence of *Myroides* spp. when present together in a mixed biofilm (Bremer *et al.*, 2001). Together with the enhanced colonization of *L. monocytogenes* in mixed-species biofilms, increased persistence of *L. monocytogenes* strains during disinfecting experiments was also observed (Bremer *et al.*, 2001). Enhanced persistence to sanitizers and disinfectants increases the risk of contamination of food products by *L. monocytogenes* in food processing plants (Gandhi and Chikindas, 2006). In addition, the presence of *L. monocytogenes* as part of biofilm communities in these environments also increases the risk of increased resistance to antibiotics by contaminating strains, which leads to difficulty in treating food-borne listeriosis (Gandhi and Chikindas, 2006). These concerns are not restricted to *L. monocytogenes* food spoilage and spread of disease through contaminated products. Other spoilage organisms have been found as part of biofilms in food processing environments (Van Houdt *et al.*, 2004; and Donlan, 2002). For example, *S. thermophilus* has been isolated from biofilms attached to the heat exchangers in milk processing equipment (Genigeorgis, 1995). Various parts of processing equipment such as gaskets, valves and dead ends in pipe systems, as well as

cracks, corners, joints and crevices, are continuously in contact with, and thus coated by organic materials providing favorable niches for bacterial attachment (Poulsen, 1999).

3.5.2 Clinical environment

The formation of biofilms in a clinical setting is also a cause of great concern. According to the Centers for Disease Control and Prevention, 70% of human bacterial infections in the Western world are caused by biofilms (Harrison *et al.*, 2005). Many of these diseases are caused by microorganisms that are common, free-living inhabitants of the human body that become virulent once sessile in biofilm communities (Netting, 2001). Clinical and public health scientists have tried to elucidate the formation and persistence of these biofilms, since the ability to withstand increased concentrations of antimicrobials, leads to augmented difficulty in treating disease. Other characteristics of biofilms that can be important in infectious diseases include: detachment of cells or biofilm aggregates, which may result in bloodstream or urinary tract infections, or emboli; an augmented production of endotoxins; and resistance to host immune system clearance (Donlan, 2002).

Biofilm-associated bacteria have been implicated in cystic fibrosis, native valve endocarditis, otitis media, periodontitis, kidney infections and chronic prostatitis (Harrison *et al.*, 2005; Donlan, 2002; and Bell, 2001). Biofilm-associated bacteria have been found attached to non-native tissues and prostheses, of which some of these surfaces (contact lenses or sutures) are easily removable and thus eradication of these infections does not require surgical removal (Bell, 2001; and Costerton *et al.*, 1999). Intravascular devices such as central venous catheters have also been found to harbour biofilms (Donlan, 2002; Elliott *et al.*, 1997; Maki, 1994; and Raad *et al.*, 1992), and to complicate this problem, it was found that human blood actually enhances the attachment and formation of these biofilms (Murga *et al.*, 2001; Espersen *et al.*, 1990; and Herrmann *et al.*, 1988). The most challenging sites to eradicate biofilm growth are endovascular devices such as prosthetic heart valves and synthetic vascular grafts (Bell, 2001). After a course of antibiotic treatment, relapses of biofilm-related infections is a common

occurrence. This often necessitates surgical removal and replacement of the infected tissue or medical device (Jefferson, 2004).

Environmental biofilms may also be sources of pathogenic bacteria that could infect humans (Bell, 2001). *Legionella* spp. are able to co-exist with bacterial and protozoal saprophytes in microbial biofilms, which colonize water-heaters and other water-handling systems, such as hospital water systems (Atlas, 1999). Colonization of hospital water-handling systems pose a threat to human health since bacteria which have broken free of these biofilms are often found to infect the lungs of patients on ventilators (Harrison *et al.*, 2005). Species of *E. meningoseptica* have been isolated from biofilm communities in sink taps of a hospital and implicated in a neonatal meningitis outbreak (Hoque *et al.*, 2001). In addition, species of the genus *Chryseobacterium* were isolated from a biofilm containing potentially pathogenic bacteria from drinking water samples in South Africa (Pavlov *et al.*, 2004).

3.5.3 Aquatic environments

3.5.3.1 Potable water systems

Bacterial biofilms are mostly found in aquatic environments, and thus other areas of concern are drinking (potable) water distribution systems (Szewzyk *et al.*, 2000; and Block, 1992). Biofilms that form on the surface of pipes and fittings are believed to act as a reservoir for the pathogens (Van der Wende *et al.*, 1989; LeChevallier *et al.*, 1987; and Camper *et al.*, 1985). Pathogens such as *E. coli* (Buswell *et al.*, 2001; Camper *et al.*, 1996; and Robinson *et al.*, 1995), *A. hydrophila* (Walker *et al.*, 1995), *Legionella pneumophila* (Murga *et al.*, 2001; Rogers *et al.*, 1994; and Colbourne *et al.*, 1984), *C. jejuni* (Buswell *et al.*, 1999) and *H. pylori* (Mackay *et al.*, 1999) have been found associated with biofilms developed from tap-water microorganisms in potable water systems. Other problems associated with multispecies biofilms formed within potable water systems include microbially-induced corrosion and unpleasant taste and odor (Kerr *et al.*, 2003).

3.5.3.2 Industrial water systems

In industrial settings, biofilms are the leading cause of microbial-influenced corrosion, and may also cause clogging of industrial pipes and filters (Pratt and Kolter, 1999; and Costerton *et al.*, 1995). Biofilm organisms were even isolated from flowing water photo-processing tanks by Elvers *et al.* (1998), where *C. indologenes* was isolated from complex multispecies consortia, which contained not only bacteria but also filamentous fungi and yeasts. Bacterial isolates displaying similarity to *C. gleum*, *C. indologenes* and *C. balustinum* have been isolated from biofilms in paper mills, where these biofilms have been shown to negatively affect machine efficiency and paper quality (Oppong *et al.*, 2003). In many countries, water reclamation is an important health and environmental issue. A constantly encountered problem in these systems is the presence of pathogens that may be harboured in biofilm communities and become a potential source of contamination (Hu *et al.*, 2003).

3.5.3.3 Aquaculture industry

Definite biofilm formation, the organisms preferring plastic surfaces, but also adhering to concrete and steel surfaces and increased resistance to tetracycline and chloramphenicol, antibiotics often used in aquacultural practices, was observed for the well-known bacterial shrimp larvae pathogen, *V. harveyi* (Karunasagar *et al.*, 1996). *M. odoratum* isolates were isolated from biofilms on weldments in a seafood processing plant where it was observed that at 15°C, *M. odoratum* enhance the survival of *L. monocytogenes* on these surfaces (Tide *et al.*, 1999). *Flavobacterium* spp. have been found to enhance the attachment and accumulation of *L. monocytogenes* (Jeong and Frank, 1994).

Mixed species biofilm communities may also play advantageous roles, as they are able to produce enzymes that degrade complex substances and may remove toxic substances from the environment. *C. gleum* has been used in a mixed species consortium, containing *A. radiobacter* and *Pseudomonas* spp. for the degradation of the pesticide pentachlorophenol (PCP) (Yu and Ward, 1996). PCP is associated with wood preservation and used as a disinfectant in the food industry and poses a significant health

hazard. The ability to degrade various toxic compounds, such as furan and phenolic compounds (Lopez *et al.*, 2004), and the insecticide and nematocide carbofuran (Bernardet *et al.*, 2006) has been described for *C. indologenes*. *C. indologenes* has been used in a mixed species consortium for the degradation of aniline and 4-chloroaniline from contaminated soil (Radianingtyas *et al.*, 2003).

The study of microorganisms in mixed-species biofilms is becoming increasingly important in order to elucidate their mechanisms of adherence and maturation into biofilm communities as a means of eradicating biofilm communities in the natural environment that could potentially harbour pathogens, or control medically important, disease-causing biofilms. The present study proposed to investigate the biofilm-forming capacity of bacterial species belonging to the genera *Chryseobacterium*, *Elizabethkingia*, *Myroides* and/or *Empedobacter* isolated from aquaculture settings. Initial adherence abilities, cell-surface properties contributing to adherence and biofilm formation, and aggregation properties were investigated, and *in vitro* flow-cell biofilm studies were conducted.

4. Experimental Philosophy

4.1 Hypothesis to be tested

It is hypothesized that *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. isolates possess the capacity to form biofilms, facilitating their existence and transmission as pathogens within aquaculture systems. It is further hypothesized that their surface hydrophobicity properties as well as their intrinsic autoaggregation and coaggregation abilities enable these isolates to bind to biotic and abiotic substrates and consequently mediate biofilm formation by these bacteria.

4.2 Objectives

- 4.2.1 To differentiate isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. by using phenotypic and molecular typing methods.

- 4.2.2 To investigate the effect of environmental cues on the degree of adherence of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. isolates.
- 4.2.3 To determine the role of motility in biofilm formation.
- 4.2.4 To determine the role of the outer membrane surface properties in biofilm formation.
- 4.2.5 To determine whether adhesion of isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. to surfaces is mediated by hydrophobic interactions or by specific receptor interactions.
- 4.2.6 To determine whether *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. biofilm formation is influenced by adherence to other bacterial cells on the substrate.
- 4.2.7 To investigate biofilm formation using flow cell assays.

4.3 Aims

The following aims will be pursued:

- 4.3.1 To characterize and identify study isolates phenotypically, biochemically and by 16S rRNA PCR-RFLP typing.
- 4.3.2 To determine whether isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. are motile by agar motility assays.
- 4.3.3 To differentiate between isolates by capsule staining.
- 4.3.4 To differentiate between isolates by random amplified polymorphic DNA (RAPD) fingerprinting, whole cell protein (WCP), and outer membrane protein (OMP) profiling.
- 4.3.5 To identify biofilm formation by isolates using the microtitre plate assay. To investigate the impact of altered temperature, nutrients, dynamic conditions, and role of spent medium on biofilm formation by isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp.
- 4.3.6 To investigate the hydrophobicity of isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. by salt aggregation test (SAT) and microbial adhesion to hydrocarbon (MATH) assays.

- 4.3.7 To determine the ability of isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. isolates to coaggregate and autoaggregate. To determine possible receptors involved in coaggregation through coaggregation reversal studies.
- 4.3.8 To investigate biofilm formation with selected isolates of *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. using flow cells under different nutrient conditions.

4.4 Questions to be answered

- 4.4.1 Do *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. isolates possess the ability to form biofilms?
- 4.4.2 Do specific environmental cues impact on biofilm formation by *Chryseobacterium*, *Elizabethkingia* and *Myroides* spp. isolates?
- 4.4.3 Does the presence of molecules involved in quorum sensing play a role in inducing biofilm formation by the study isolates?
- 4.4.4 Do the study preferentially adhere to hydrophobic or hydrophilic surfaces, and if so to what degree?
- 4.4.5 Does motility and the presence of a capsule influence biofilm formation? How important are these structures in mediating initial adherence to surfaces?
- 4.4.6 Does surface hydrophobicity play a role in the ability of these organisms to form biofilms?
- 4.4.7 Is there interstrain variation with respect to the degree of surface hydrophobicity?
- 4.4.8 Are the isolates able to coaggregate with members of their respective genus as well as with other microorganisms? Do the study isolates display a greater inclination to autoaggregate or coaggregate?

CHAPTER TWO

Polyphasic identification and characterization of *Chryseobacterium*, *Elizabethkingia*, *Myroides* and *Empedobacter* spp. isolated from aquaculture systems

2.1 Introduction

The genera *Chryseobacterium*, *Elizabethkingia*, *Myroides* and *Empedobacter* belong to the family *Flavobacteriaceae* which was first proposed by Jooste *et al.* (1985), and later described and validated by Reichenbach (1992) and Holmes (1997), respectively. These genera are continually changing, as new species are isolated, described and the biochemical, physiological and molecular properties of individual strains and species belonging to these genera are investigated (Bernardet *et al.*, 2006). They have been differentiated extensively at a phenotypic level (Bernardet *et al.*, 2006; and Hugo *et al.*, 2005).

Members of the genera *Chryseobacterium* and *Elizabethkingia* are aerobic, non-motile, Gram-negative rods that display bright yellow to orange pigmentation, a result of flexirubin pigments (Vandamme *et al.*, 1994; and Reichenbach *et al.*, 1981). The absence of gliding motility and the presence of flexirubin pigments differentiate these genera from other genera in the family *Flavobacteriaceae* (Bernardet *et al.*, 2006). *E. meningoseptica* is non-pigmented or slightly yellow-beige coloured, but production of the yellow pigment is strain-dependent (Bernardet *et al.*, 2002). Colonies are translucent, circular, convex to low convex, smooth, and shiny with entire edges (Vandamme *et al.*, 1994). Prolonged incubation of certain species of both genera produce extracellular slimy substances which are responsible for the mucoid consistency of the colonies (Kim *et al.*, 2005a; Kim *et al.*, 2005b; Young *et al.*, 2005; and Kämpfer *et al.*, 2003). *Chryseobacterium* and *Elizabethkingia* spp. grow at 25°C and 30°C, while growth at 37°C varies among species of the genus *Chryseobacterium*, and very poor growth or no growth occurs at 5 °C, and certain species may grow at 42°C (Bernardet *et al.*, 2006; Kämpfer *et al.*, 2003; Bernardet *et al.*, 2002; and Vandamme *et al.*, 1994). All species display catalase and oxidase activities (Vandamme *et al.*, 1994), and gelatin hydrolysis (Bernardet *et al.*, 2002). The

production of proteases (gelatin- and casein-degrading proteases) is considered an important virulence factor of some members of the family *Flavobacteriaceae* (Bernardet and Bowman, 2005). These proteases degrade components of muscle, cartilage and connective tissue, which include actin and myosin, elastin, type IV collagen, fibrinogen, fibronectin, gelatin and laminin (Bernardet and Bowman, 2005). Acid production from glucose, indole production and starch hydrolysis varies among the *Chryseobacterium* spp. (de Beer *et al.*, 2006; Park *et al.*, 2006; Weon, *et al.*, 2006; de Beer *et al.*, 2005; Kim *et al.*, 2005a; Bernardet *et al.*, 2002; Campbell and Williams, 1951; and Harrison, 1929). Most *Chryseobacterium* and *Elizabethkingia* spp. isolates exhibit a high tolerance to NaCl, with growth observed on nutrient agar of up to 5% NaCl (Bernardet *et al.*, 2006).

Members of the genera *Myroides* and *Empedobacter* are Gram-negative, non-motile rods which are lightly pigmented due to flexirubin pigmentation and produce a fruity odour (Bernardet *et al.*, 2002). Both genera are strictly aerobic, produce oxidase and catalase, grow on McConkey agar, and degrade gelatin (Hugo *et al.*, 2005). They grow at 25°C, 30°C and 37°C, but growth does not take place at 5°C and 42°C (Hugo *et al.*, 2005). The genus *Empedobacter* differs from the genus *Myroides* by acid production from glucose, the production of indole, and the variable hydrolysis of starch, characteristics for which *Myroides* spp. are negative. *Empedobacter* spp. colonies are pinpoint, low convex, circular, smooth and shiny, with entire edges. Four colony types have been described for *Myroides* spp.: (1) effuse, spreading colonies with raised, shiny centers and dull, matt, spreading edges that becomes smooth and shiny after long incubation periods; (2) resembles colony type 1, but colonies are smaller; (3) smooth, shiny, and convex with no spreading edges; and (4) mucoid (Holmes *et al.*, 1979). Variable tolerance of salt concentrations has been observed for *Myroides* spp. isolates, with tolerance of up to 9% (w/v) NaCl (Yoon *et al.*, 2006). Although it is very difficult to distinguish between *Myroides* spp. isolates (Hugo *et al.*, 2005), *M. odoratus* and *M. odoratimimus* differ in carbon source assimilation (Biotype 100 assays) and oxidation (Biolog GN MicroPlate assays), and demonstrate a slight difference in their G + C content (Vancanneyt *et al.*, 1996). *M. odoratimimus* has significantly higher amounts of cellular fatty acids, and thus cellular fatty acid composition has been used to distinguish between the two *Myroides* spp. (Vancanneyt *et al.*, 1996).

Extensive use of molecular typing methods have been described for the typing of pathogenic organisms responsible for disease outbreaks in animals in veterinary, farming and environmental settings (Figueiro *et al.*, 2005; Arias *et al.*, 2004; Szczuka and Kaznowski, 2004; Cloeckert *et al.*, 2003; Coquet *et al.*, 2002b; Michel *et al.*, 2002; Crump *et al.*, 2001; Madetoja *et al.*, 2001; Madsen and Dalgaard, 2000; Triyanto and Wakabayashi, 1999; Triyanto *et al.*, 1999; Chakroun *et al.*, 1997; and Bernardet *et al.*, 1996). Members of the genera *Chryseobacterium*, *Elizabethkingia*, *Myroides*, and *Empedobacter* have not been typed extensively using molecular typing methods. However, Colding *et al.* (1994) used ribotyping to type clinical *Elizabethkingia* spp. isolates for epidemiological studies, while DNA macrorestriction was used for the differentiation of *E. meningoseptica* isolates (Sader *et al.*, 1995). Pulsed-field gel electrophoresis was used to demonstrate that human and environmental strains of *E. meningoseptica* isolated during an outbreak of meningitis in a neonatal intensive care unit belonged to the same strain (Hoque *et al.*, 2001). Pulsed-field gel electrophoresis (PFGE) and infrequent-restriction site PCR were suggested to be very effective tools for the discrimination of *E. meningoseptica* strains isolated during epidemiological investigations of a meningitis outbreak (Lin *et al.*, 2004). A clear differentiation between *Chryseobacterium* and *Elizabethkingia* spp. was reported through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Hugo *et al.*, 1999). Similar results were obtained by Bernardet *et al.* (2005), who demonstrated delineation of clusters of species belonging to the genus *Chryseobacterium* through random amplified polymorphic DNA PCR (RAPD) analysis and whole-cell protein (WCP) analysis. RAPD fingerprinting was also used to distinguish between a cluster of pathogenic *E. meningoseptica* strains and *E. meningoseptica* isolates from diverse geographic areas (Chiu *et al.*, 2000). Amongst members of the genera *Chryseobacterium* and *Elizabethkingia*, several examples of 16S rRNA gene sequence similarity values well above 97% between members of different species may be found (Li *et al.*, 2003). Sequence similarities of up to 99% between *Chryseobacterium* spp. have been described (Li *et al.*, 2003), indicating the overall high similarity and the resulting difficulty in delineating new species in this genus.

Isolates belonging to the genus *Myroides* have been typed by DNA macrorestriction with restriction enzymes *Sma*I and *Sst*II and subsequent PFGE which allowed for discrimination between different strains of *M. odoratimimus* (Yagci *et al.*, 2000). Arbitrarily-primed PCR techniques yielded identical profiles for all *M. odoratimimus* strains during these investigations and was, therefore, not a good discriminatory tool in this case (Yagci *et al.*, 2000).

Antimicrobial susceptibility testing is a phenotypic typing method that has been frequently used for members of these genera, due to their involvement in opportunistic human infections. Resistance phenotypes are important to identify trends in susceptibility or resistance and aid in the treatments prescribed for disease in both human and veterinary medicine. The high levels of resistance demonstrated by *Chryseobacterium* and *Elizabethkingia* spp. to a wide range of antimicrobial agents and their unusual susceptibility patterns have been useful in their preliminary identification (Bernardet *et al.*, 2006). *Chryseobacterium* spp. isolates are resistant to many antimicrobial agents including extended-spectrum penicillins, first- and second-generation cephalosporins, and carbapenems (β -lactam antibiotics) as well as aminoglycosides, tetracyclines, and chloramphenicol (Schreckenberger, 1998). Sensitivity to piperacillin/tazobactam has been reported (Lin *et al.*, 2003). Resistance to penicillin and polymyxin is used in the identification of *E. meningoseptica* (Schreckenberger, 1998). Similarly, isolates belonging to both the *Myroides* and *Empedobacter* genera are resistant to a wide range of antimicrobial agents, particularly β -lactams (penicillins, cephalosporins, aztreonam, and carbapenems), aminoglycosides, tetracyclines, quinolones and trimethoprim-sulfamethoxazole, but successful treatment with ciprofloxacin has been reported (Yagci *et al.*, 2000; Hsueh *et al.*, 1995; Macfarlane *et al.*, 1985; Strandberg *et al.*, 1983; and Holmes *et al.*, 1979).

There have been increasing reports of fish disease from which *Chryseobacterium* and *Elizabethkingia* spp. have been isolated, and since few antibiotics are currently authorized for fish disease control, most of these multiple-drug resistant species are able to evade eradication following treatment. This leads to an increase in their prevalence, higher aquaculture losses and an increase in possible transmission to humans where they may act as opportunistic pathogens (Michel *et al.*, 2005). Successful antibiotic therapy of

fish-associated *Chryseobacterium* and *Elizabethkingia* spp. was reported by treatment with oxolinic acid and associated sulfonamides by Michel *et al.* (2005).

OMPs may serve as adhesins anchored on the outer membrane surface of Gram-negative bacteria (Ofek *et al.*, 2003). These adhesins play an important role in virulence of bacteria as they have been found to mediate intimate adhesion to the target cell by bacteria and are often associated with invasion of the target cell or formation of a lesion at the adhesion site (Ofek *et al.*, 2003). As these porins and other OMPs of bacteria have an important function in the recognition of specific binding sites and adherence of organisms, they serve not only as a phenotypic typing tool but may also reveal important information on the biofilm-forming ability of bacteria (Donlan, 2002; Wang *et al.*, 2002; Decostere *et al.*, 1999; and Stickler, 1999).

A diversity of yellow-pigmented bacteria were isolated on sampling of fish from various South African aquaculture sources. To examine and specifically identify the diversity of members of the family *Flavobacteriaceae* and the genera *Chryseobacterium*, *Elizabethkingia*, *Empedobacter* and *Myroides* specifically, isolates were characterized phenotypically as well as by a diversity of molecular techniques.

2.2 Materials and Methods

2.2.1 Bacterial isolates and growth conditions

Isolates were obtained from tilapia, trout and salmon specimens presenting with external lesions or from internal organs of fish displaying gill necrosis, skin ulcers and systemic disease (Table 2.1). Thirty-four *Chryseobacterium* and *Elizabethkingia* spp. isolates and five *Myroides* and *Empedobacter* spp. isolates were tentatively selected on the basis of colonial characteristics (colony color, odour and morphology). Reference strains *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), *C. indoltheticum* (ATCC 27950), *C. joostei* (LMG 18212), *C. meningosepticum* (NCTC 10016), and *M. odoratus* (NCTC 11036) were included in the study. Study isolates and reference strains were maintained on enriched Anacker and Ordal's agar (EAOA) (Anacker and Ordal, 1959) at ambient temperature

(21 °C ± 2°). For long-term storage, cultures were placed in 80% glycerol and enriched Anacker and Ordal's broth (EAOB) and stored at -80 °C.

2.2.2. Physiological Characterization

Presence of yellow to orange pigmentation was confirmed by overnight (O/N) growth of study isolates on EAOA plates. Adherence of colonies to agar, and sticky or mucoid consistency of colonies were determined visually and documented. Isolates were streaked onto EAOA plates in triplicate to determine the ability to grow at different temperatures (5 °C, 26 °C, 37 °C and 42 °C).

Motility assays were carried out on modified casitone yeast (CY) agar medium (Jooste *et al.*, 1985) containing: 3 g/l casitone, 1 g/l yeast extract, 1 g/l CaCl₂·2H₂O, 15 g/l agar (pH 7.2 adjusted with KOH). Ten µl of O/N cultures equivalent to a 0.5 McFarland standard were stab-inoculated with a sterile inoculating needle. Isolates were inoculated in triplicate and incubated at room temperature for 5 days. Motility was determined by measuring the migration zones according to Kempf and McBride (2000): Strong gliding ability (zone ≥ 2.5 cm), weak gliding ability (zone < 2.5 cm) and no gliding ability was documented when no spreading zones were observed.



Table 2. 1 *Chryseobacterium* and *Elizabethkingia* spp. and *Myroides* and *Empedobacter* spp. isolates, host species, geographical location and date of isolation.

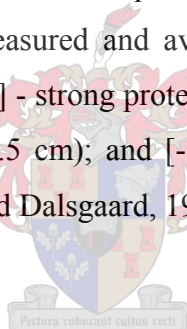
Strains	Species of fish (samle)	Origin	Date
MY1	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
MY2	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
MY2B	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
MY3	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
MY3B	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH1	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH1B	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH2	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH2B	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH3	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH4	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH4B	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH5	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH6	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH7	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH8	Blue tilapia (<i>Oreochromis mossambicus</i>)	Stellenbosch, RSA	2003
CH9	Blue tilapia (<i>Oreochromis mossambicus</i>) lateral fin	Stellenbosch, RSA	2003
CH10	Blue tilapia (<i>Oreochromis mossambicus</i>) brain	Stellenbosch, RSA	2003
CH11	Blue tilapia (<i>Oreochromis mossambicus</i>) skin	Stellenbosch, RSA	2003
CH12	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Stellenbosch, RSA	2003
CH13	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Stellenbosch, RSA	2003
CH14	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Franschhoek, RSA	2003
CH15	Rainbow trout (<i>Oncorhynchus mykiss</i>) eggs	Stellenbosch, RSA	2003
CH16	Rainbow trout (<i>Oncorhynchus mykiss</i>) eye	Malawi, Africa	2003
CH17	Rainbow trout (<i>Oncorhynchus mykiss</i>) eye	Malawi, Africa	2003
CH18	Rainbow trout (<i>Oncorhynchus mykiss</i>) eye	Malawi, Africa	2003
CH19	Rainbow trout (<i>Oncorhynchus mykiss</i>) eye	Malawi, Africa	2003
CH21	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Stellenbosch, RSA	2003
CH22	Rainbow trout (<i>Oncorhynchus mykiss</i>) skin	Stellenbosch, RSA	2003
CH23	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Stellenbosch, RSA	2003
CH24	Rainbow trout (<i>Oncorhynchus mykiss</i>) liver	Stellenbosch, RSA	2003
CH25	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Franschhoek, RSA	2003
CH26	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Franschhoek, RSA	2003
CH27	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Franschhoek, RSA	2003
CH28	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Franschhoek, RSA	2003
CH29	Atlantic salmon (<i>salmo salar</i>) gill	Stanford, RSA	2005
CH30	Atlantic salmon (<i>salmo salar</i>) gill	Stanford, RSA	2005
CH33	Rainbow trout (<i>Oncorhynchus mykiss</i>) gill	Stellenbosch, RSA	2005
CH34	Rainbow trout (<i>Oncorhynchus mykiss</i>) fin	Stellenbosch, RSA	2005

2.2.3. Biochemical characterization

The following standard biochemical tests were performed for initial identification of the isolates: Gram-staining (Hucker modification), oxidase and catalase production, indole production, Voges-Proskauer tests, citrate utilization, starch hydrolysis, growth on McConkey agar (Harvey and Prescott, 1996), growth in the presence of 5% NaCl and

9% NaCl (Hugo *et al.*, 2005, and Yoon *et al.*, 2006). The presence of glucosamine capsular material was determined by Congo red accumulation (Crump *et al.*, 2001), where the ability to absorb the red pigment was investigated by growth on EAOB plates containing 0.003 g/l Congo Red, which was recorded over a 2 to 5 d period.

Proteolytic activity of the test isolates was determined by gelatin and casein hydrolysis. Gelatin hydrolysis was investigated by stab-inoculation of test isolates into gelatin deep-tubes, incubation for 3 days at 30 °C and examined for gelatin liquefaction following 30 min refrigeration to eliminate false-positive results (Kaminski and Ferroni, 1980). Growth on Tryptone Yeast Extract Salts (TYES) agar (tryptone, 4 g/l; yeast extract, 0.4 g/l; CaCl₂.2H₂O, 0.2 g/l; MgSO₄.7H₂O, 0.5 g/l; and agar, 10 g/l) supplemented with 2% or 10% skim milk was used to test casein hydrolysis (Cepeda *et al.*, 2004; and Madsen and Dalsgaard, 1998). Ten µl of O/N broth cultures equivalent to a 2 McFarland standard were inoculated in triplicate, plates incubated at 30 °C for one week, and zone diameters were measured and averaged for each isolate. Proteolytic activity was classified as follows: [+] - strong proteolytic activity (zone > 2.5 cm); [(+)] - weak proteolytic activity (zone ≤ 2.5 cm); and [-] - no proteolytic activity (no zones) (Cepeda *et al.*, 2004; and Madsen and Dalsgaard, 1998).



2.2.4 Genotypic typing

2.2.4.1 Isolation of Genomic DNA

Genomic DNA of study isolates and the reference strains was isolated by the CTAB/NaCl mini-prep protocol (Ausubel *et al.*, 1989). O/N EAOB cultures were harvested by centrifugation and cell pellets resuspended in 567 µl TE buffer [10 mM Tris-HCl, and 1 mM EDTA, (pH 8)]. Thirty µl SDS and 3 µl 20 mg/ml proteinase K were added and incubated at 37 °C for 1 h. After the addition of 100 µl of 5 M NaCl and 80 µl CTAB/NaCl [10% CTAB, and 0.7 M NaCl], the mixtures were incubated at 65 °C for 10 min. Genomic DNA in the mixtures were extracted by phenol/chloroform/isoamyl alcohol extraction, precipitated using isopropanol and pellets were washed with 70% ethanol. Resulting pellets were resuspended in TE buffer and stored at -20 °C.

2.2.4.2 16S rRNA gene PCR-RFLP

Genomic DNA of the study isolates and reference strains was subjected to PCR for the amplification of the 1.5 kb 16S rRNA gene sequence using primers 16S-F (5'-AGTTTGATCCTGGCTCAG -3') and 16S-R (5'-TACCTTGTTACGACTTCACCCCA-3') described by Heyndrickx *et al.* (1996). Twenty-five µl reaction volumes comprised of 1.5 µM of each primer, 100 µM dNTP's (Roche, Germany), 1.5 mM MgCl₂, 1U SuperTherm DNA polymerase (JMR Holdings, UK), 1 × reaction buffer and 100 ng genomic DNA. Amplifications were performed in a MJ Mini™ Gradient Thermal Cycler (BioRad, USA) under the following amplification conditions: 94 °C for 3 min, followed by 35 amplification cycles of DNA denaturation at 94 °C for 30s, primer annealing at 52 °C for 1 min, and extension at 72 °C for 1 min, and a final extension step of 72 °C for 8 min. PCR products were subjected to electrophoresis in a 1.5% 1 × TAE - agarose gel (40 mM Tris base, 20 mM glacial acetic acid, and 2 mM EDTA), stained with ethidium bromide and viewed by UV transillumination. The O'Gene Ruler 100 bp Plus DNA ladder (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada) was included in the gels, as a molecular weight marker.

Resulting 1.5 kb 16S rDNA amplicons were digested with restriction enzymes *CfoI*, *MspI* and *TaqI* (Roche, Germany), respectively. Respective restriction fragments were then subjected to polyacrylamide gel electrophoresis in 8% polyacrylamide gels [10.64 ml of 30% acrylamide (29 g acrylamide, 1 g N,N'-methylenebisacrylamide, and ddH₂O to 100 ml), 21.08 ml ddH₂O, 8 ml 5 × TBE (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA), 20% ammonium persulfate, and 14 µl N,N,N',N'-tetramethylethylenediamine (TEMED)] in 1 × TBE-buffer at 5 mA for 15 hours. The O'Gene Ruler 100 bp DNA ladder (Fermentas, Canada) was included as a molecular weight marker. Restriction patterns were examined visually for differences in number and sizes of fragments. Differences in molecular weight of fragments were estimated using UVIDOC V.97 (UVItec, UK) software.

2.2.4.3 Random Amplified DNA Polymerase Chain Reaction (RAPD-PCR)

Genomic DNA of the study isolates and reference strains was subjected to RAPD-PCR analysis using primers P1 (5'-CGCCCTGCCC-3') and P2 (5'CTGCTGGGAC-3'), respectively (Bernardet *et al.*, 2005). Reaction mixtures (25µl) contained 1 × Exsel-therm Taq DNA polymerase reaction buffer, 1U Exsel-therm Taq DNA polymerase (JMR Holdings, UK), 1.5mM MgCl₂, 100 µM dNTP's (Roche, Germany), 1.5 µM of either oligonucleotide primer, and 100 ng template DNA. Cycle parameters included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52 °C for 1 min, 72°C for 1 min, and finally 8 min at 72°C in a MJ Mini™ Gradient Thermal Cycler (BioRad, USA). Negative controls containing no template DNA were included in all experiments, and in order to ensure reproducibility RAPD amplifications were consistently performed using the same thermal cycler. Additionally, isolates MY1 and CH1 were repeatedly amplified on separate occasions to confirm reproducibility of RAPD profiles.

PCR products were subjected to electrophoresis in a 1.5% 1 × TAE - agarose gel, stained with ethidium bromide and viewed by UV transillumination. The O'GeneRuler 100 bp DNA ladder plus (Fermentas, Canada) was used as a size marker. Band sizes of RAPD amplicons were calculated using UVIDOC V.97 (UVItec, UK). Fingerprints were visually examined for differences in number of fragments amplified, differences in molecular weight and intensity of PCR fragments. RAPD profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

2.2.5 Phenotypic typing

2.2.5.1 Antimicrobial Susceptibility of test isolates

Antibiotic susceptibility to 25 antimicrobial agents was determined using antibiotic disks (Mast Diagnostics and Oxoid, UK), on Mueller-Hinton (MH) agar plates (Biolab, Merck

Gauteng) following NCCLS protocols (NCCLS, 2002). The panel of antibiotics used included: amikacin (AK-30), ampicillin (AP-10), amoxicillin (A-10), augmentin (AU-30), azithromycin (ATH-15), ceftazadime (CAZ-30), cefoxitin (FOX-30), ceftriaxone (CRO-30), cefuroxime (CXM-30), chloramphenicol (C-30), ciprofloxacin (CIP-5), cotrimoxazole (TS-25), erythromycin (E-15), gentamicin (GM-10, GM-120), imipenem (IMI-10), nalidixic acid (NA-30), norfloxacin (NOR), ofloxacin (OFX-5), oxacillin (OX-1), piperacillin/tazobactam (TZP), sulphamethoxazole (SMX-25), streptomycin (S-10), tetracycline (T-25), and trimethoprim (TM-1.25). Isolates were grown overnight in EAOB, the turbidity of the cell suspensions were adjusted to that equivalent to a 0.5 McFarland standard and used to inoculate MH agar plates, which were incubated for 24 h at 26 °C. Bacterial strains *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 25923 were used as antimicrobial susceptibility testing controls, according to NCCLS recommendations. Testing was done in duplicate and resistance profiles (resistant, intermediate, or susceptible) were assigned after measuring average zone diameters using NCCLS breakpoints (NCCLS, 2002).

MAR index values (a/b , where 'a' represents the number of antibiotics the isolate was resistant to and 'b' represents the total number of antibiotics the isolate was tested against) were calculated for all isolates. When isolates are exposed to high-risk sources of contamination originating from humans or animals, where antibiotics are often used a MAR index value higher than 0.2 is observed. When antibiotics are seldom or never used a MAR index value less than or equal to 0.2 is observed (Krumperman, 1985).

2.2.5.2 Whole Cell Protein analysis

Fifty ml of O/N EAOB cultures were centrifuged at 12000 rpm for 15 min. Cells were washed with 10 ml PBS (pH7), centrifuged at 12000 rpm for 15 min and resuspended in 10 ml PBS buffer. WCP preparations were prepared by sonication in a Sonicator™ Cell Disruptor (Heat Systems-Ultrasonics Inc, USA), and 2 ml aliquots were stored at -20 °C.

Fifty µl of the protein preparations were solubilised in 20 µl 2 × sample buffer [25 ml 4 × Tris-Cl/SDS (pH 6.8), 20 ml glycerol, 4 g SDS, 2 ml 2-mercaptoethanol, and 1 mg

bromophenol blue, 100 ml ddH₂O] and heated for 5 min at 100 °C. Preparations were subjected to electrophoresis in 14% polyacrylamide gels by SDS-PAGE at 5 A for 14 h. A PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada) was used as protein marker.

SDS-PAGE gels were stained by silver staining, using a modified protocol described by Tsai and Frasch (1982). SDS-PAGE gels were immersed in a fixing solution (50% methanol, 12% acetic acid and 0.0185% formaldehyde) for 1 h. This was followed by two 10 min washes in washing solution (50% ethanol), pretreatment in 2% sodium thiosulphate solution for 5 min, and 3 × 30 s washes in sterile ddH₂O. Gels were subsequently stained in silver nitrate solution (1 ml of 0.2% silver nitrate and 0.028% formaldehyde) for 10 min, and washed twice in sterile ddH₂O. To visualize protein bands, gels were immersed in developer (6% sodium carbonate, 0.0185% formaldehyde and 0.14 mg sodium thiosulphate). After sufficient developing, stop solution (50% methanol and 12% acetic acid) was added.

Gels were photographed with a FujiFilm FinePix 3800 digital camera (Fuji Photo Film Co., LTD. Japan) and fixed in cellophane in a Gel Air Dryer (BioRad, USA). Protein band sizes were calculated using UVIDOC V.97 (UVItec, UK). Protein profiles were visually examined for differences in number, molecular weight and intensity of protein bands. WCP profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

2.2.5.3 Outer Membrane Protein analysis

A modification of the method described by Benedí and Martínez-Martínez (2001) was used to prepare OMPs with sodium-lauryl-sarcosine (SLS). Fifty ml of O/N EAOB cultures were centrifuged at 12000 rpm for 15 min. Cells were washed with 10 ml PBS (pH 7), centrifuged at 12000 rpm for 15 min and resuspended in 10 ml PBS. An OmniRuptor 400 Ultrasonic Homogenizer (OMNI International Inc, USA) was used to sonicate the cells, followed by centrifugation for 10 min at 6000 rpm. Supernatants were

centrifuged in a L7-65 Ultracentrifuge (Beckman Instruments Inc, USA) at 37000 rpm at 4 °C for 1 h. Resulting pellets were resuspended in 2 ml of a 2% SLS-PBS solution and incubated at room temperature for 30 min. Suspensions were centrifuged at 37000 rpm for 45 min at 4°C. Partially purified OMP preparations were prepared by resuspension of the pellets in a 1% SLS PBS solution and centrifugation at 4 °C for 40 min at 37000 rpm. Pellets were resuspended in ddH₂O and stored at -20 °C.

Protein preparations were solubilised in 20 µl 2 × sample buffer and heated for 5 min at 100 °C. This was followed by electrophoresis in 12% polyacrylamide gels by SDS-PAGE at 5 A for 14 h. The protein marker PageRuler™ unstained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada) was included in every electrophoresis run. Gels were stained with the modified silver staining method, as described previously (Tsai and Frasch 1982).

Gels were photographed with a FujiFilm FinePix 3800 digital camera (Fuji Photo Film Co., LTD. Japan) and fixed in cellophane in a Gel Air Dryer (Bio-Rad laboratories, USA). Protein band sizes were calculated using UVIDOC V.97 (UVItec, UK). Protein profiles were visually examined for differences in number, molecular weight and intensity of protein bands. OMP profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

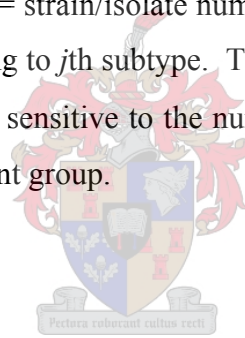
2.2.6 Typeability, reproducibility and discrimination ability of typing methods

Data obtained by the typing techniques were evaluated according to the following criteria: typeability, reproducibility and discrimination. Typeability refers to the ability of a typing technique to give an unambiguous positive result for each given isolate, thus, giving a percentage of distinct bacterial isolates which can be assigned a positive type (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). Reproducibility refers to a typing technique's ability to produce the same result for a given isolate on repeated testing. Therefore, reproducibility is the percentage of isolates giving the same result on repeated testing. Over time, minor variation might be observed in types of isolates and,

therefore, reproducibility also refers to the typed attribute's stability over time (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). Discriminatory power refers to a typing technique's ability to differentiate epidemiologically unrelated isolates (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). Ideally, the technique should assign each isolate to a different type (Tenover *et al.*, 1997). Traditional phenotypic typing methods usually show lower discriminatory power compared to molecular methods (Tenover *et al.*, 1997). The use of a single numerical index of discrimination (D), based on the probability that two unrelated isolates sampled from a test population will be placed in distinct typing groups, can be calculated using the Simpson's index of diversity (Hunter and Gaston, 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=0}^{j=s} n_j(n_j - 1)$$

where, D = numerical index; N = strain/isolate number in sample; s = subtype number; n_j = strain/isolate number belonging to j th subtype. The desired discrimination index of a typing scheme is $\geq 90\%$, which is sensitive to the number of groups defined by a typing scheme and the size of the dominant group.



2.3 Results

2.3.1 Physiological and biochemical identification of isolates of *Myroides* and *Empedobacter* spp.

Phenotypic and biochemical test results obtained for the *Myroides* and *Empedobacter* (MY) and *Chryseobacterium* and *Elizabethkingia* (CH) spp. isolates are summarized in Table 2.2. The isolates were then grouped for further analysis based on their phenotypic and biochemical test results.

Group I contained all of the *Myroides* and *Empedobacter* spp. isolates. Isolates were all Gram-negative (Fig. 2.1) and produced the characteristic fruity odour described for *Myroides* spp. (Hugo *et al.*, 2005). Colonies initially resembled *Myroides* spp. colony type 3 as smooth, shiny, convex colonies with no spreading edges, but were transformed to colony type 4 as the colonies became mucoid after 7 days. Isolates differed from the *Myroides* and *Empedobacter* spp. description (Hugo *et al.*, 2005) by their inability to

grow on McConkey agar. Weak proteolytic activity was observed as the isolates were only able to partially degrade gelatin, as opposed to complete gelatin hydrolysis described previously by Hugo *et al.* (2005), and partial degradation of casein. With regards to differentiating between the *Myroides* and *Empedobacter* genera, study isolates shared characteristics of the genus *Myroides* by being negative for the production of glucose and their inability to degrade starch, and to the genus *Empedobacter* by their indole-positive phenotype. The *Myroides* and *Empedobacter* spp. isolates displayed very thick capsules (Fig. 2.2) and no motility was detected. Cells displayed the characteristic red flexirubin pigment following NaOH exposure (Fig. 2.3). Growth in the presence of 5% NaCl was observed for all isolates (Fig. 2.4), but no growth occurred in the presence of 9% NaCl.

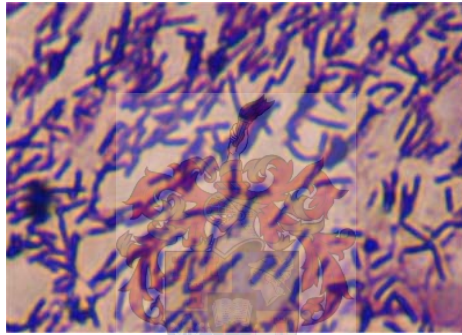


Figure 2. 1 Light microscope image of Gram-negative staining reaction displayed by *Myroides* sp. isolate MY1 ($\times 1000$ magnification).

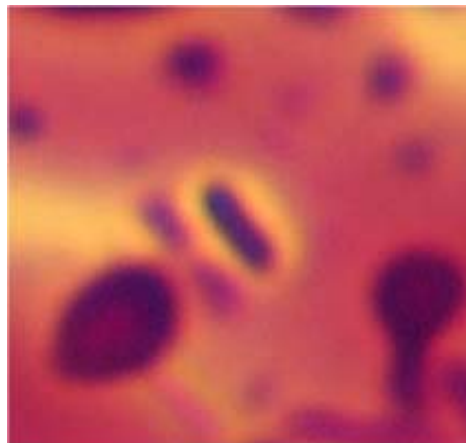


Figure 2. 2 Light microscope image of thick capsular material surrounding *Myroides* sp. isolate MY1 following negative staining ($\times 1000$ magnification).



Figure 2. 3 Release of characteristic red flexirubin pigment from *Myroides* sp. isolate MY1 cells following exposure to 10 N NaOH.



Figure 2. 4 Growth of *Myroides* sp. isolate MY2B on nutrient agar supplemented with 5% NaCl.

Table 2.2 Phenotypic and biochemical Characteristics of *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates.

Experiments	Myroides isolates					Chryseobacterium isolates																
	MY1	MY2	MY2B	MY3	MY3B	CH1	CH1B	CH2	CH2B	CH3	CH4	CH4B	CH5	CH6	CH7	CH8	CH9	CH10	CH11	CH12	CH13	
Physiological characteristics																						
Colony morphology*	A	A	A	A	A	1	1	1	2 [†]	1	1	1	1	1	1	1	3	2	2	2	1	
Adherence to agar [#]	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	-	-	+	
<i>Growth following temperature variation</i>																						
5°C	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+					
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	
42°C [§]	-	-	-	-	-	(+)	(+)	+	+	-	(+)	-	(+)	-	-	-	+	-	-	-	-	
Biochemical characteristics																						
Gram Reaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Flexirubin pigment production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Indole Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Voges-Proskauer																						
glucose Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Starch utilization	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	
Growth on McConkey agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Growth on Nutrient agar containing 5% (w/v) NaCl	+	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	
Growth on Nutrient agar containing 9% (w/v) NaCl [¥]	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
Proteolytic activity																						
Gelatin Hydrolysis	(+)	(+)	(+)	(+)	(+)	+	(+)	+	(+)	+	+	+	+	+	+	+	+	(+)	(+)	(+)	+	
Casein Hydrolysis	(+)	(+)	(+)	(+)	(+)																	
Congo Red uptake	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Capsule (Light microscope investigation) [‡]	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	(+)	+	-	(+)	-	(+)	(+)	
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

* Colony morphology – *Myroides* and *Empedobacter* spp. isolates: (A) light yellow, small shiny convex colonies with smooth edges;
 - *Chryseobacterium* and *Elizabethkingia* spp. isolates: (1) Orange, big shiny convex colonies with smooth edges; (†1) larger colonies; (2) orange, small colonies with smooth edges; (†2) lighter pigmentation; (3) deeper orange, larger convex colonies with smooth edges; (4) yellow colonies with delayed flexirubin pigmentation, very small convex colonies with smooth edges

Adherence to agar - Mucoid, stringy consistency of colonies
 § += positive; - = negative; (+) = weak positive
 ¥ ND = not determined; + = positive; - = negative; (+) = weak positive for 9% (w/v) NaCl
 ‡ Capsule classification - + = Thick capsule; - = No capsule; (+) = Thin capsule

Table 2.2 (Continued) Phenotypic and biochemical characteristics of *Chryseobacterium* and *Elizabethkingia* spp. isolates.

Experiments	Chryseobacterium isolates																	
	CH14	CH15	CH16	CH17	CH18	CH19	CH21	CH22	CH23	CH24	CH25	CH26	CH27	CH28	CH29	CH30	CH33	CH34
Physiological characteristics																		
Colony morphology*	2	2	2	2	2	2	1 [‡]	1	2	2	1	1	4	2	1	1	2	1
Adherence to agar [#]	+	-	-	-	-	-	+	+	-	+	+	+	+	+	-	-	-	-
Growth following temperature variation																		
5°C			-	-	-	-	+	-	-	+	+	+	+	+	+	+		
37°C	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42°C [§]	-	-	(+)	-	+	-	+	+	+	+	(+)	+	+	+	+	+	+	+
Biochemical characteristics																		
Gram Reaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flexirubin pigment production	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole Production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-Proskauer																		
glucose Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch utilization	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Growth on McConkey agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on Nutrient agar containing 5% (w/v) NaCl	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-
Growth on Nutrient agar containing 9% (w/v) NaCl [¥]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Proteolytic activity																		
Gelatin Hydrolysis	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-	-	-	(+)	(+)	(+)	(+)
Casein Hydrolysis																		
Congo Red uptake	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capsule (Light microscope investigation) [‡]	(+)	(+)	(+)	-	-	(+)	(+)	-	+	(+)	(+)	(+)	-	(+)	+	-	-	(+)
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Colony morphology – *Myroides* and *Empedobacter* spp. isolates: (A) light yellow, small shiny convex colonies with smooth edges;
- *Chryseobacterium* and *Elizabethkingia* spp. isolates: (1) Orange, big shiny convex colonies with smooth edges; (†1) larger colonies; (2) orange, small colonies with smooth edges; (†2) lighter pigmentation; (3) deeper orange, larger convex colonies with smooth edges; (4) yellow colonies with delayed flexirubin pigmentation, very small convex colonies with smooth edges

Adherence to agar - Mucoid, stringy consistency of colonies

§ + = positive; - = negative; (+) = weak positive

¥ ND = not determined; + = positive; - = negative; (+) = weak positive for 9% (w/v) NaCl

‡ Capsule classification - + = Thick capsule; - = No capsule; (+) = Thin capsule

2.3.2 Physiological and biochemical identification of *Chryseobacterium* and *Elizabethkingia* spp. isolates

While the *Myroides* spp. isolates appeared phenotypically and biochemically homogenous, differences were observed between the different *Chryseobacterium* and *Elizabethkingia* spp. isolates (Table 2.2). Four distinct colony morphology phenotypes were observed for the study isolates (Fig. 2.5): (1) Orange, big shiny convex colonies with smooth edges; (†1) larger colonies; (2) orange, small convex colonies with smooth edges; (†2) lighter pigmentation; (3) deeper orange, larger convex colonies with smooth edges; and (4) yellow, very small convex colonies with smooth edges, delayed flexirubin pigmentation (Table 2.2). All of the *Chryseobacterium* spp. and *Elizabethkingia* spp. isolates were Gram-negative rods (Fig. 2.6), and only two of the isolates (isolates CH10, CH16) did not display the characteristic flexirubin pigmentation (Table 2.2; Fig. 2.7) following NaOH exposure. All of the isolates were positive for catalase, oxidase and indole production, and negative for glucose fermentation and citrate utilization as well as growth on McConkey agar (Table 2.2). None of the *Chryseobacterium* isolates were motile. Capsule staining revealed isolate variability with respect to presence or absence and intensity of capsular material observed (Table 2.2).

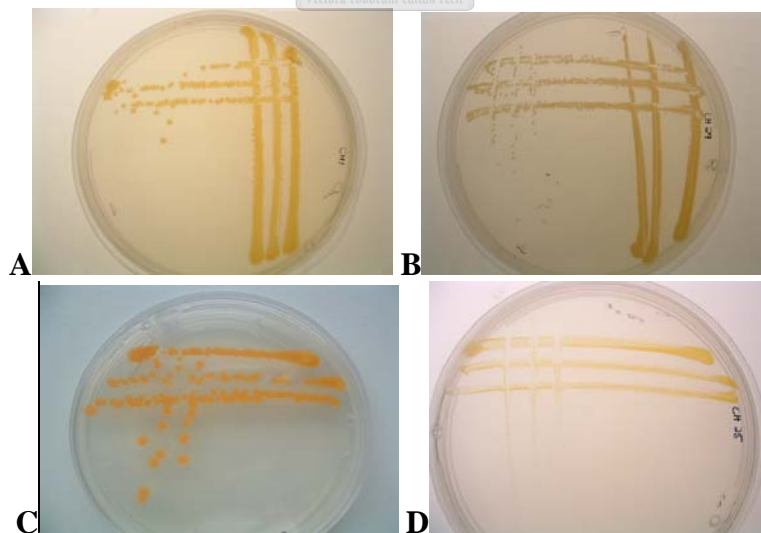


Figure 2. 5 Colony morphologies displayed by *Chryseobacterium* and *Elizabethkingia* spp. isolates: (A) - Orange, big shiny convex colonies with smooth edges; (B) - orange, small convex colonies with smooth edges; (C) - deeper orange, larger convex colonies with smooth edges; and (D) - yellow, very small convex colonies with smooth edges.

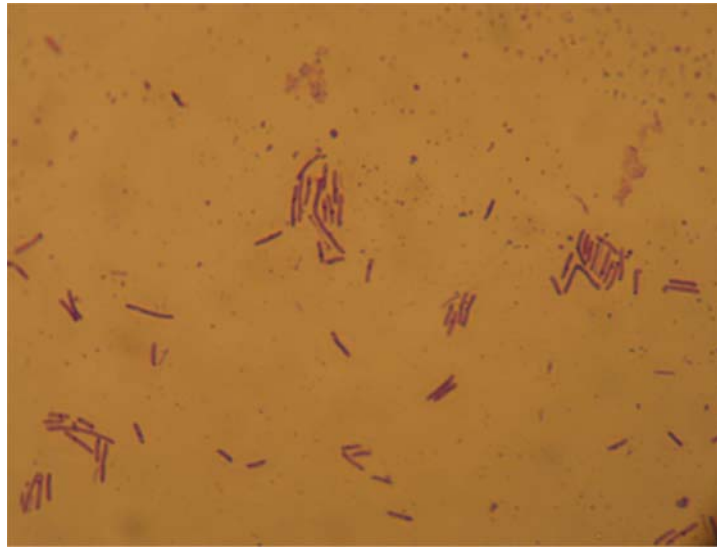


Figure 2. 6 Light microscope image of Gram-negative staining reaction displayed by *Chryseobacterium* and *Elizabethkingia* spp. isolate CH8 ($\times 1000$ magnification).



Figure 2. 7 Release of characteristic red flexirubin pigment from *Chryseobacterium* and *Elizabethkingia* spp. isolates CH8 cells following exposure to 10 N NaOH.

The *Chryseobacterium* and *Elizabethkingia* spp. isolates were grouped based on their variable phenotypic and biochemical test results (Table 2.3). Group 2a contained 6 isolates which differed from group 2b only by the ability of the former to grow at 42 °C. The isolates clustered into groups 3 and 4 (b and c) were unable to utilize starch. Group 3a isolates differed from group 3b by not being able to grow at 37 °C. Isolates in group 4 were able to grow in the presence of 5% NaCl (Fig. 2.8.) and included group 4a which were unable to grow at 42 °C and group 4b which were able to grow at these high temperatures, but could not hydrolyse starch. Group 4c differed from group 4b by delayed production of the flexirubin pigment, since the colonies only displayed their characteristic colour after 7 days of incubation. Group 5 consisted of two isolates which

did not produce the flexirubin pigment, and were further subdivided into groups 5a and 5b, where the former was unable to utilize starch and grow at 37 °C.

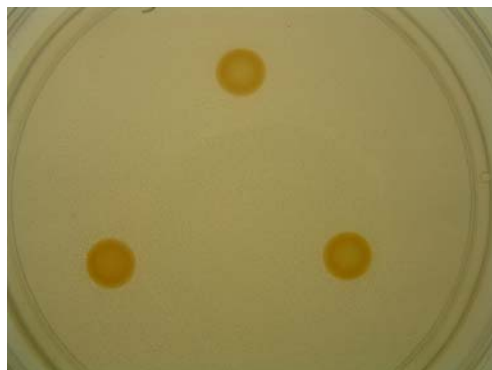


Figure 2. 8 Growth of *Chryseobacterium* and *Elizabethkingia* spp. isolates CH3 in the presence of 5% NaCl.

Table 2. 3 Groupings of *Chryseobacterium* and *Elizabethkingia* spp. isolates based on variations in phenotypic and biochemical test results.

Group	Isolates	Differentiating phenotypic/ biochemical characteristics
<u>Myroides spp. isolates</u>		
Group I	MY1, MY2, MY2B, MY3, MY3B	Positive for indole production and growth on McConkey agar; Growth on 5 % NaCl
<u>Chryseobacterium/Elizabethkingia spp. isolates</u>		
Group 2		
2a	CH1, CH1B, CH2, CH4, CH5, CH9	Growth at 42 °C
2b	CH6, CH7, CH8, CH15, CH17, CH19	No growth at 42 °C
Group 3		<u>No starch utilization</u>
3a	CH11, CH12, CH13, CH14	No growth at 37 °C
3b	CH2B, CH18, CH21, CH22, CH29, CH30, CH33, CH34	Growth at 37 °C
Group 4		<u>Growth in the presence of 5 % NaCl</u>
4a	CH3, CH4B	No growth at 42 °C; Starch and gelatin hydrolysis
4b	CH24, CH25, CH25, CH28	Growth at 42 °C; No starch or gelatin hydrolysis
4c	CH23, CH27	Delayed production of flexirubin pigment Growth at 42 °C; No starch or gelatin hydrolysis
Group 5		<u>No flexirubin pigmentation</u>
5a	CH10	No starch hydrolysis No growth at 37 °C
5b	CH16	Starch hydrolysis; Growth at 37 °C

2.3.3 Restriction Fragment Length Polymorphic (RFLP) PCR analysis

TaqI, *CfoI* and *MspI* restriction, respectively, of 16S rRNA gene amplicons of *Myroides* and *Empedobacter* spp. isolates were not sufficiently discriminatory (Figs. 2.9 – 2.11). All five isolates displayed identical profiles following restriction with *CfoI* (C-I), *MspI* (M-I) and *TaqI* (T-I) (Table 2.4). *CfoI* profiles consisted of 3 fragments ranging in size from 1114 bp to 52 bp (Fig. 2.9). *MspI* and *TaqI* restriction patterns consisted of 4 fragments each, ranging in size from 897 bp to 43 bp (Fig. 2.10) and 611 bp to 88 bp (Figs. 2.11), respectively. *CfoI* and *MspI* profiles obtained were identical to that obtained for the reference *M. odoratus* (NCTC 11036) strain (Table 2.4). However, isolates displayed a *TaqI* profile which was variable to that of the reference strain (Table 2.4; Figs. 2.11 - 2.12)

Eleven profiles (T-1 to T-11) were obtained following *TaqI* restriction of the *Chryseobacterium* and *Elizabethkingia* spp. study isolates and reference strains (Table 2.4, Figs. 2.12 - 2.16). Profiles consisted of 3 - 6 fragments ranging in size from 43 bp to 847 bp (Table 2.4). Groups T-1 and T-2 contained 11 and 12 isolates, respectively, and these profiles corresponded to that of reference strains *C. joostei* (LMG 18212) and *C. gleum* (NCTC 11432), respectively (Table 2.4; Fig 2.12). Two study isolates clustered with *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb) in group T-3.

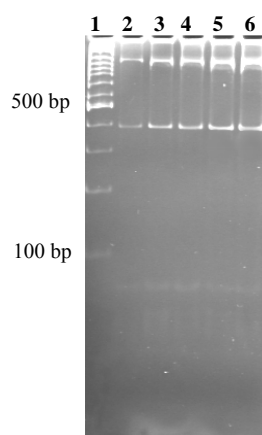


Figure 2.9 Polyacrylamide gel electrophoresis of restriction fragments of *Myroides* and *Empedobacter* spp. isolates following digestion of the 1500 bp 16S rRNA amplicon with *CfoI*. Lane 1: O'GeneRuler 100 bp DNA ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-6: isolates MY1, MY2, MY2B, MY3, MY3B, respectively.

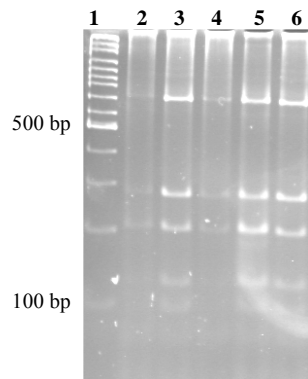


Figure 2. 10 Polyacrylamide gel electrophoresis of restriction fragments of *Myroides* and *Empedobacter* spp. isolates following digestion of the 1500 bp 16S rRNA amplicon with *MspI*. Lane 1: O'GeneRuler 100 bp DNA ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-6: isolates MY1, MY2, MY2B, MY3, MY3B, respectively.



Figure 2. 11 Polyacrylamide gel electrophoresis of restriction fragments of *Myroides* and *Empedobacter* spp. isolates following digestion of the 1500 bp 16S rRNA amplicon with *TaqI*. Lane 1: O'GeneRuler 100 bp DNA ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada), lanes 2-6: isolates MY1, MY2, MY2B, MY3, MY3B, respectively.

One study isolate each in groups T-4 and T-11, (Table 2.4) clustered with the reference *C. indoltheticum* (ATCC 27950) and *E. meningoseptica* (NCTC 10016) strains, respectively. None of the study isolates clustered with *C. balustinum* (NCTC 11212) (Table 2.4). Isolate CH16 appeared to be untypeable by *TaqI* restriction and did not yield a *TaqI* profile despite repeated attempts ($\times 3$) to obtain a restriction profile (Table 2.4).

Groups T-1 to T-4 could be grouped into the same cluster as restriction of the 16S rRNA of the isolates belonging to this group displayed similar restriction fragments

differing from each other by very few base pairs (Table 2.4). Although belonging to this cluster, it was possible to differentiate between reference cultures *C. joostei*, *C. gleum*, *C. indologenes* and *C. indoltheticum*, respectively, with *TaqI* restriction of the 16S rRNA. No other clusters were observed following *TaqI* restriction analysis, and one fragment of approximately 49 bp was common to 50% of the profiles.

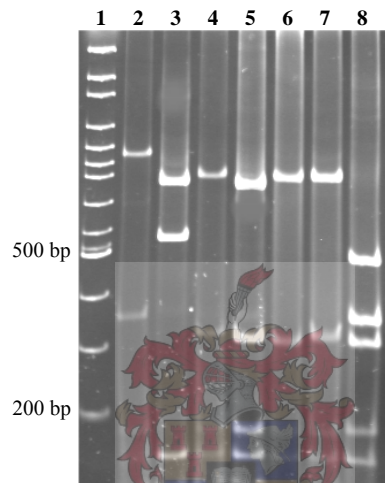


Figure 2. 12 Poly-Acrylamide gel electrophoresis of restriction fragments of reference strains following digestion of the 1500 bp 16S rRNA amplicon with *TaqI*. Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 2-8: reference cultures *M. odoratus* (NCTC 11036), *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), *C. indoltheticum* (ATCC 27950), *C. joostei* (LMG 18212), and *E. meningoseptica* (NCTC 10016), respectively.

Table 2. 4 16S rRNA amplicon PCR-RFLP profiles of yellow-pigmented study isolates and selected *Myroides*, *Chryseobacterium* and *Elizabethkingia* spp. reference strains.

Restriction Enzyme	Profiles				Isolates	Number of isolates
	No. of patterns	No. of fragments	Subtype	Size of fragments (bp)		
TaqI	2 (<i>Myroides/Empedobacter</i>)	4	T-I	897; 355; 185; 43	MY1, MY2, MY2B, MY3, MY3B	5
		4	T-II	993; 363; 175; 142	<i>M. odoratus</i>	1
	11 (<i>Chryseobacterium/Elizabethkingia</i>)	4	T-1	790; 314; 173; 129; 89	CH1, CH1B, CH13, CH15, CH17, CH18, CH19, CH22, CH27, CH29, CH30,	12
		5	T-2	812; 325; 176; 134; 91	<i>C. joostei</i>	11
		5	T-3	759; 319; 173; 133; 93	CH2, CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9, CH10, <i>C. gleum</i>	11
		5	T-4	793; 313; 171; 130; 87	CH11, CH21, <i>C. indologenes</i>	3
		3	T-5	517; 200; 47	CH14, <i>C. indoltheticum</i>	2
		4	T-6	847; 363; 191; 46	CH12	1
		4	T-6	847; 363; 191; 46	CH23, CH24	3
		5	T-7	642; 351; 212; 145; 48	CH25, CH26, CH28	3
		6	T-8	820; 600; 542; 358; 171; 43	CH33	1
		4	T-9	313; 199; 147; 49	CH34	1
		4	T-10	780; 570; 143; 99	<i>C. balustinum</i>	1
	6	T-11	490; 363; 328; 183; 156; 55	CH2B, <i>E. meningoseptica</i>	2	
	0	T-0		CH16	1	
CfoI	1 (<i>Myroides/Empedobacter</i>)	3	C-I	1114; 391; 52	MY1, MY2, MY2B, MY3, MY3B, <i>M. odoratus</i>	6
	13 (<i>Chryseobacterium/Elizabethkingia</i>)	6	C-1	900; 191; 148; 108; 88; 46	CH1, <i>C. gleum</i> , <i>C. indologenes</i> , <i>C. indoltheticum</i>	4
		5	C-2	870; 238; 145; 86; 49	CH1B, CH2, CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9, CH10, CH11, CH13, CH15, CH17, CH18, <i>E. meningoseptica</i>	17
		5	C-3	836; 243; 208; 147; 46	CH2B	1
		4	C-4	688; 221; 212; 141	CH19, CH22	2
		3	C-5	674; 332; 212	CH21	1
		5	C-6	533; 329; 212; 126; 43	CH23	1
		4	C-7	677; 410; 234; 54	CH24	1
		4	C-8	513; 404; 143; 53	CH25, CH26, CH28	3
		4	C-9	864; 238; 222; 143	CH27, CH29, CH30	3
		5	C-10	389; 335; 319; 235; 180	CH33	1
		2	C-11	230; 195	CH34	1

Table 2.4 (continued). 16S rRNA amplicon PCR-RFLP profiles of yellow-pigmented study isolates and selected *Myroides*, *Chryseobacterium* and *Elizabethkingia* spp. reference strains.

Restriction Enzyme	Profiles				Isolates	Number of isolates
	No. of patterns	No. of fragments	Subtype	Size of fragments (kbp)		
CfoI	11 (<i>Chryseobacterium/Elizabethkingia</i>)	4	C-12	615; 417; 354; 193	<i>C. balustinum</i>	1
		5	C-13	900; 242; 149; 131; 109	<i>C. joostei</i>	1
		0	C-0		CH12, CH14, CH16	3
	1 (<i>Myroides/Empedobacter</i>)	5	M-I	611; 398; 206; 127; 88	MY1, MY2, MY2B, MY3, MY3B, <i>M. odoratus</i>	6
MspI	20 (<i>Chryseobacterium/Elizabethkingia</i>)	4	M-1	309; 200; 125; 55	CH1	1
		4	M-2	646; 262; 147; 67	CH1B, CH3, CH5	3
		3	M-3	772; 283; 211	CH2, CH4, CH4B, CH8, CH10, CH17, CH18	7
		5	M-4	651; 481; 342; 129; 57	CH2B	1
		3	M-5	778; 308; 228	CH6	1
		5	M-6	763; 300; 223; 141; 105	CH7, <i>C. gleum</i>	2
				712; 279; 208; 130; 94	CH9, CH19, CH22, CH27	5
		5	M-7		<i>C. indologenes</i> , <i>C. indoltheticum</i>	5
		2	M-8	529; 206	CH12	1
		5	M-9	580; 457; 232; 175; 132	CH13	1
		2	M-10	574; 357	CH14	1
		4	M-11	707; 277; 214; 132	CH15	1
		4	M-12	800; 273; 205; 124	CH11, CH21, <i>C. joostei</i>	3
		4	M-13	453; 276; 176; 159	CH23	1
		4	M-14	596; 220; 174; 165	CH24	1
		3	M-15	607; 282; 172	CH25, CH26, CH28	3
		4	M-16	753; 276; 204; 84	CH29, CH30	3
		6	M-17	558; 303; 294; 221; 144; 101	CH33	1
		3	M-18	289; 220; 205	CH34	1
		6	M-19	647; 388; 213; 139; 116; 99	<i>C. balustinum</i>	1
4	M-20	704; 535; 110; 103	<i>E. meningoseptica</i>	1		
	0	M-0		CH16	1	

Thirteen restriction profiles (C-1 to C-11) (Figs. 2.17 – 2.20) were generated following restriction with *CfoI* (Table 2.4). Profiles consisted of 2 - 6 fragments ranging in size from 900 bp to 53 bp (Table 2.4). Isolate CH1 was differentiated from isolates clustered into group C-2 (Table 2.4).

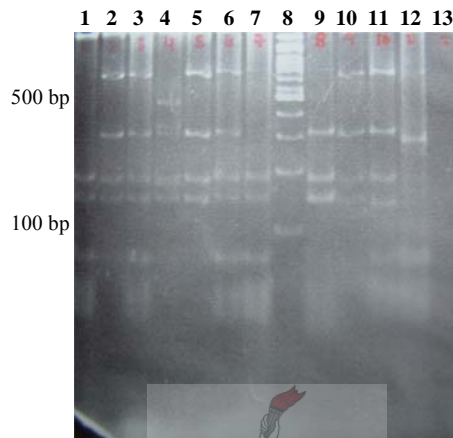


Figure 2. 13 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. study isolates following digestion of the 1500 bp 16S rRNA amplicon with *TaqI*. Lanes 1-7: CH1, CH1B, CH2, CH2B, CH3, CH4, CH4B; lane 8: O^oGeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 9-13: CH8, CH11, CH13, CH14, and CH16, respectively.

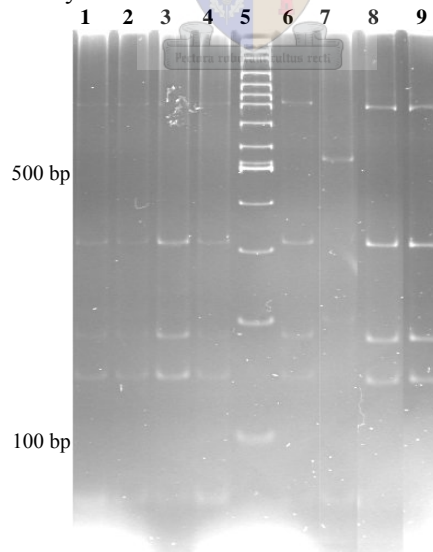


Figure 2. 14 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. study isolates following digestion of the 1500 bp 16S rRNA amplicon with *TaqI*. Lanes 1-4: CH5, CH6, CH7, CH8; lane 5: O^oGeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 6-9: CH10, CH12, CH15, and CH17, respectively.

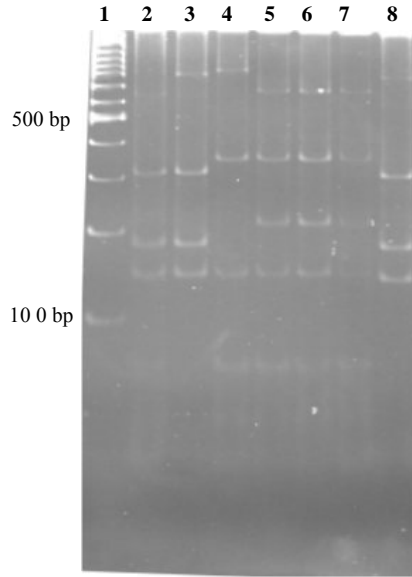


Figure 2. 15 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. study isolates following digestion of the 1500 bp 16S rRNA amplicon with *TaqI*. Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 2-8: CH9, CH21, CH23, CH25, CH26, CH28, and CH30, respectively.

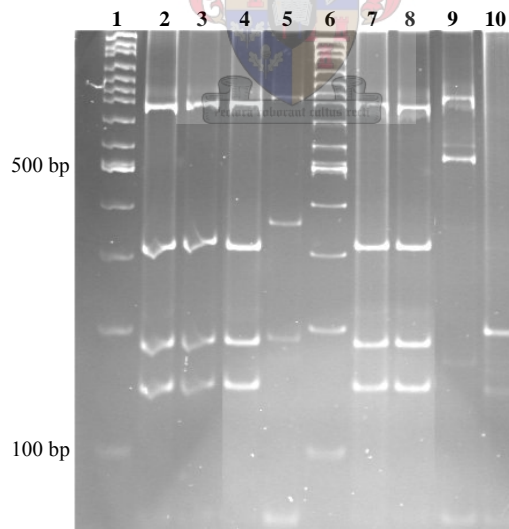


Figure 2. 16 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. study isolates following digestion of the 1500 bp 16S rRNA amplicon with *TaqI*. Lanes 1 and 6: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-5: CH18, CH19, CH22, CH24; and lanes 7-10: CH27, CH29, CH33, and CH34, respectively.

Although *CfoI* restriction allowed for the differentiation of *E. meningoseptica* (NCTC 10016) and study isolate CH2B, the *E. meningoseptica* (NCTC 10016) reference strain was clustered into a larger group of isolates (group C-2) which contained isolates belonging to the *TaqI* *C. joostei* / *C. gleum* complex (Table 2.4). Three isolates, CH12, CH14 and CH16, could not be typed by *CfoI* restriction, although isolates CH12 and CH14 were differentiated by *TaqI* restriction (Table 2.4). *CfoI* PCR-RFLP analysis did not discriminate between reference strains *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), and *C. indoltheticum* (ATCC 27950) (Table 2.4; Fig. 2.17). *C. joostei* was grouped into an individual group separating this strain from the test isolates it had grouped with by *TaqI* restriction analysis. No clusters were observed in the *CfoI* restriction patterns, and no prominent bands were observed for all the profiles, although two fragments of approximately 145 bp and 49 bp were common to 45% and 54% of the profiles, respectively.

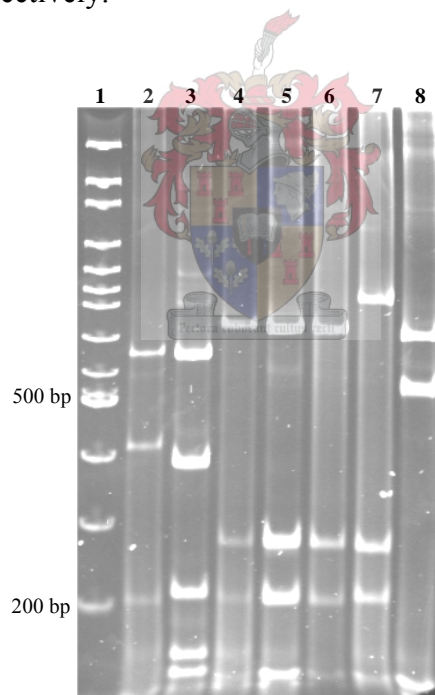


Figure 2. 17 Polyacrylamide gel electrophoresis of restriction fragments of reference strains following digestion of the 1500 bp 16S rRNA amplicon with *CfoI*. Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-8: standard cultures *M. odoratus* (NCTC 11036), *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), *C. indoltheticum* (ATCC 27950), *C. joostei* (LMG 18212), and *E. meningoseptica* (NCTC 10016), respectively.

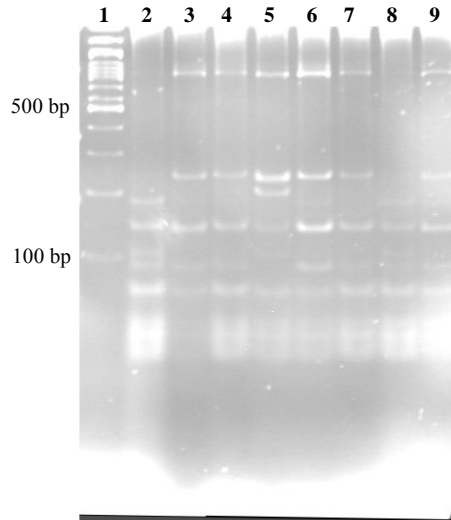


Figure 2. 18 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. isolates following digestion of the 1500 bp 16S rRNA amplicon with *Cfo*I. Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 2-9: CH1, CH1B, CH2, CH2B, CH3, CH4, CH4B, and CH5, respectively.

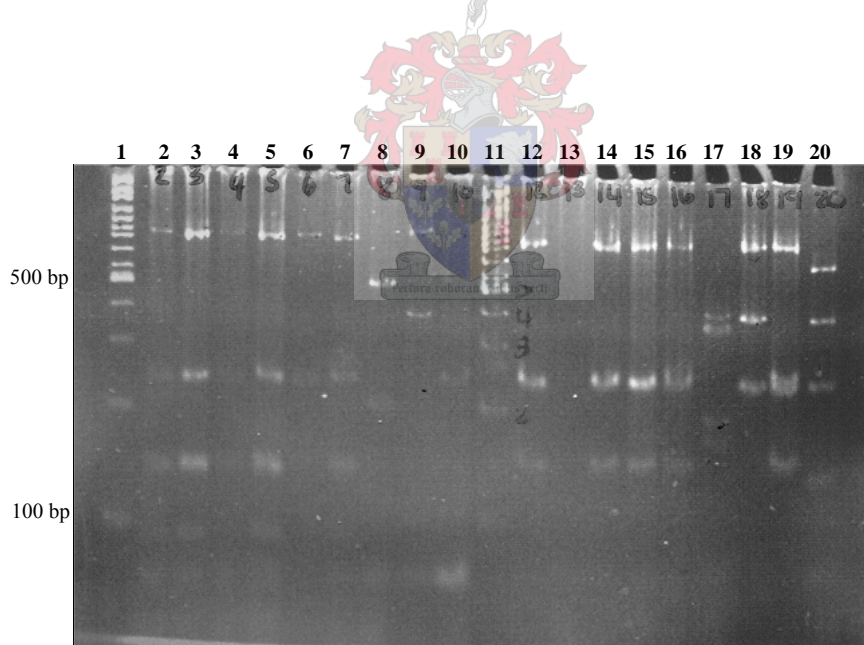


Figure 2. 19 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. isolates following digestion of the 1500 bp 16S rRNA amplicon with *Cfo*I. Lanes 1 and 11: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-10: CH6, CH7, CH8, CH9, CH10, CH11, N/A, CH13, N/A; and lanes 12-20: CH15, CH16, CH17, CH18, CH19, N/A, CH21, CH22, and CH23, respectively. (N/A – band sizes not applicable as isolates were not included in study)

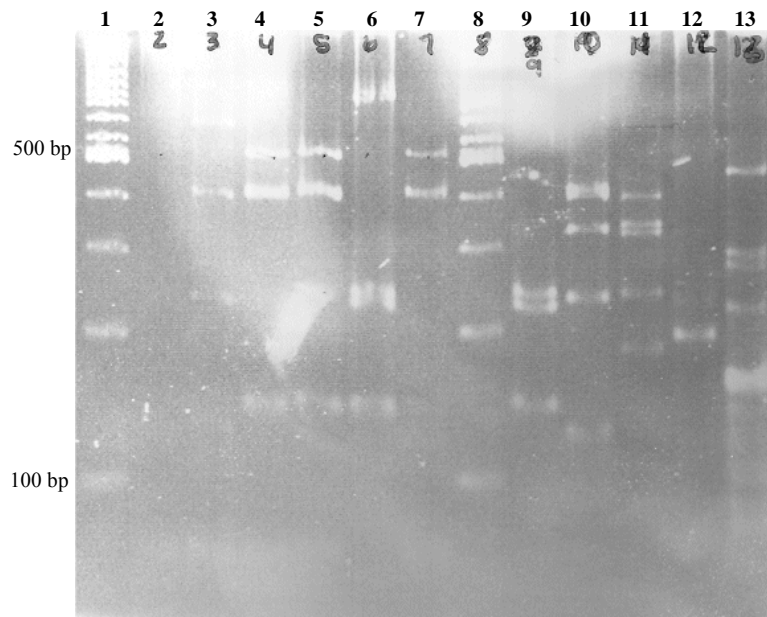


Figure 2. 20 Polyacrylamide gel electrophoresis of restriction fragments of *Chryseobacterium* and *Elizabethkingia* spp. isolates following digestion of the 1500 bp 16S rRNA amplicon with *CfoI*. Lanes 1 and 8: The O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-7: CH14, CH24, CH25, CH26, CH27, CH28; and lanes 9-13: CH29, CH30, CH33, CH34, N/A, respectively. (N/A – band sizes not applicable as isolates were not included in study)

Twenty profiles (M-1 to M-20) were obtained following *MspI* restriction (Figs. 2.21-2.25), with profiles consisting of 2 - 6 fragments ranging in size from 800 bp to 55 bp (Table 2.4). With *CfoI*, study isolate CH1 (group M-1) grouped individually, and did not cluster with any reference strains (Table 2.4). *MspI* allowed for the differentiation of isolates clustered into groups C-2 (*CfoI*) and T-1/2 (*TaqI*) (Table 2.4). Both *CfoI* and *MspI* allowed for the differentiation of isolates clustered into groups T-1 and T-6 by *TaqI* restriction (Table 2.4). Study isolates CH25, CH26 and CH28 displayed identical profiles with all three restriction enzymes (Table 2.4).

One cluster was observed following *MspI* restriction, this cluster contained 4 groups (M-3, M-5, M6 and M16) which shared three similar fragments of approximately 767 bp, 292 bp, and 217 bp in size differing by a few base pairs (Table 2.4). Group M-6 that formed part of this cluster contained two extra fragments of 141 bp and 105 bp, and group M-16 also part of this cluster contained one 84 bp fragment in addition to the

clustering fragments (Table 2.4). Two fragments of approximately 125 bp and 207 bp in size were observed for 45% of the profiles (Table 2.4).

None of the reference strains were consistently identical with specific study isolates following restriction with the three respective restriction enzymes. However, study isolate CH7 and reference strain *C. gleum* (NCTC 11432) shared similar restriction patterns following *TaqI* and *MspI* restriction (groups T-2 and M-5) (Table 2.4). Reference strain *E. meningoseptica* (NCTC 10016) grouped by itself with *MspI* restriction (M-20) (Table 2.4; Fig. 2.21). The 16S rRNA amplicons of study isolate CH16 and reference strain *C. balustinum* (NCTC 11212) were non-typeable with *MspI*, as well as *TaqI* and *CfoI* (Table 2.4).

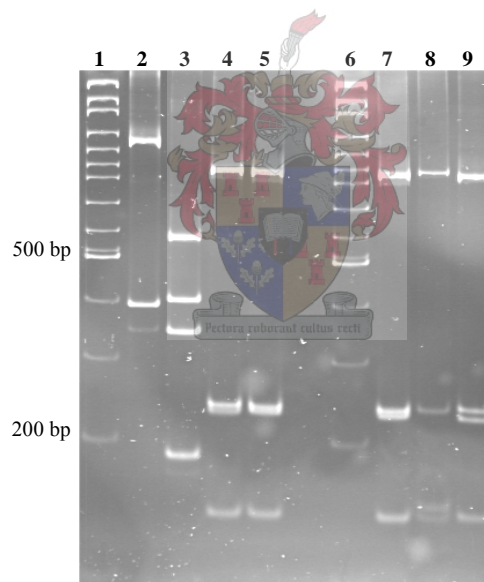


Figure 2. 21 Polyacrylamide gel electrophoresis of restriction fragments of reference strains following digestion of the 1500 bp 16S rRNA amplicon with *MspI*. Lanes 1 and 6: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-5: reference strains *M. odoratus* (NCTC 11036), *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb); and lanes 7-9: *C. indoltheticum* (ATCC 27950), *C. joostei* (LMG 18212), and *E. meningoseptica* (NCTC 10016), respectively.

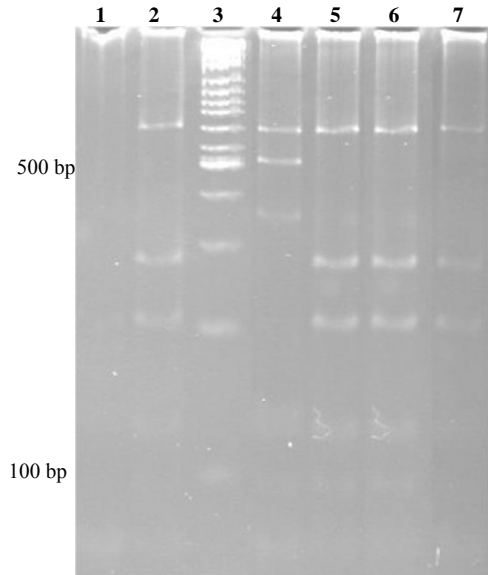


Figure 2. 22 Polyacrylamide gel electrophoresis of restriction fragments of study isolates following digestion of the 1500 bp 16S rRNA amplicon with *MspI*. Lanes 1-2: CH1, CH1B; lane 3: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 4-7: CH2B, CH2, CH3, and CH5, respectively.

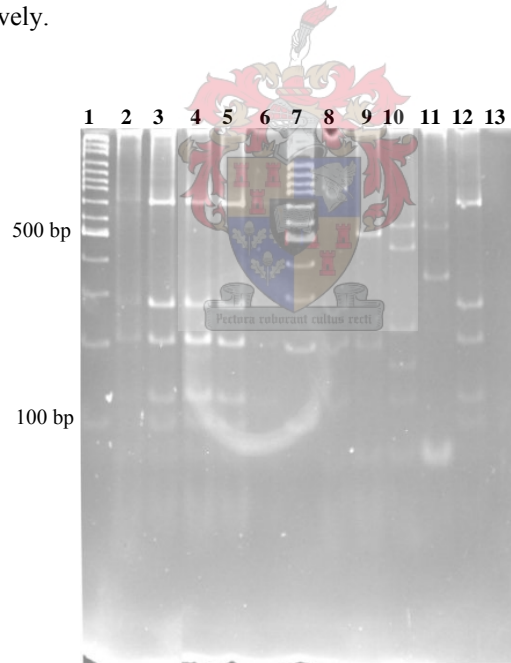


Figure 2. 23 Polyacrylamide gel electrophoresis of restriction fragments of study isolates following digestion of the 1500 bp 16S rRNA amplicon with *MspI*. Lanes 1 and 7: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 2-6: CH6, CH7, CH8, CH9, CH10; and lanes 8-13: CH11, CH12, CH13, CH14, CH15, and CH16, respectively.

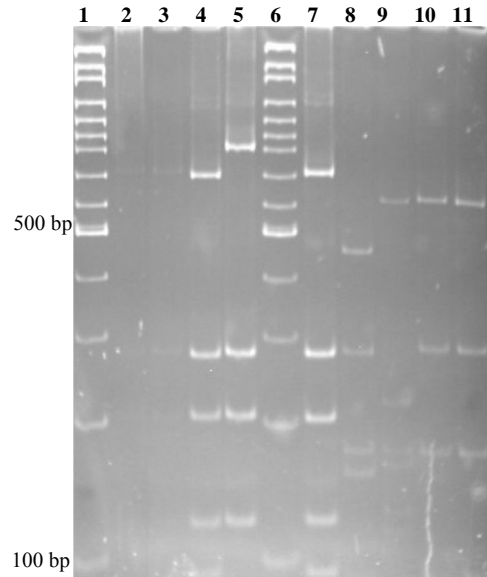


Figure 2. 24 Polyacrylamide gel electrophoresis of restriction fragments of study isolates following digestion of the 1500 bp 16S rRNA amplicon with *MspI*. Lanes 1 and 6: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-5: CH4, CH4B, CH19, CH21; and lanes 7-11: CH22, CH23, CH24, CH25, CH26, CH17, and CH18, respectively.

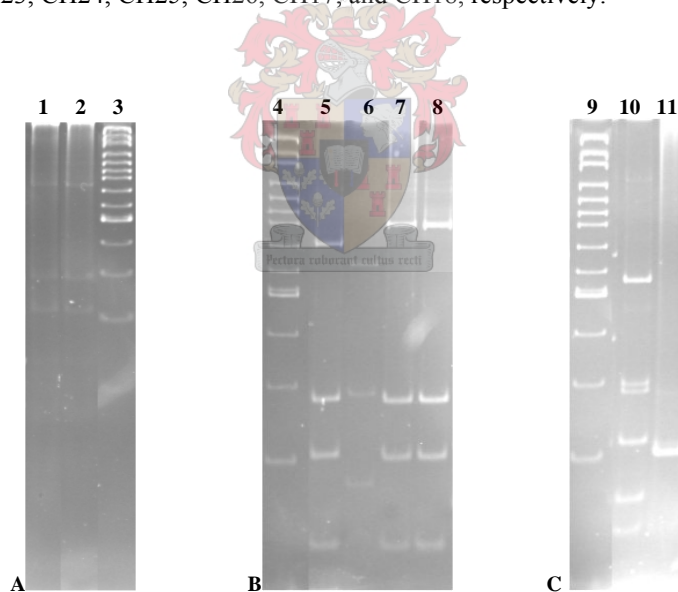


Figure 2. 25 Polyacrylamide gel electrophoresis of restriction fragments of study isolates following digestion of the 1500 bp 16S rRNA amplicon with *MspI*. Lanes 3(A), 4 (B), and 9(C): O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); (A): lanes 1-2: (B): lanes 5-8: CH27, CH28, CH29, CH30; and (C): lanes 10-11: CH33 and CH34, respectively.

2.3.4 Random amplification of polymorphic DNA (RAPD) PCR analysis

RAPD fingerprinting with both the primers P1 and P2 produced single patterns, respectively, for the *Myroides* and *Empedobacter* spp isolates (Fig. 2.26). The P1 primer generated 3 bands ranging in size from approximately 470 bp to 1151 bp (Table 2.5; Fig. 2.26A). Three fragments, ranging in size from approximately 1889 bp to 909 bp were generated with primer P2 (Table 2.5; Fig. 2.26B). It was not possible to differentiate between species using these RAPD primers.

High reproducibility was observed for isolate MY1 strains, and although the concentration of genomic DNA of reference strain *M. odoratus* (NCTC 11036) was identical for both the P1 and P2 RAPD reactions, no visible fragments were obtained with the P1 primer (Fig. 2.27). Therefore, it was not possible to identify the study isolates to species-level by P1 RAPD fingerprinting (Fig. 2.27).

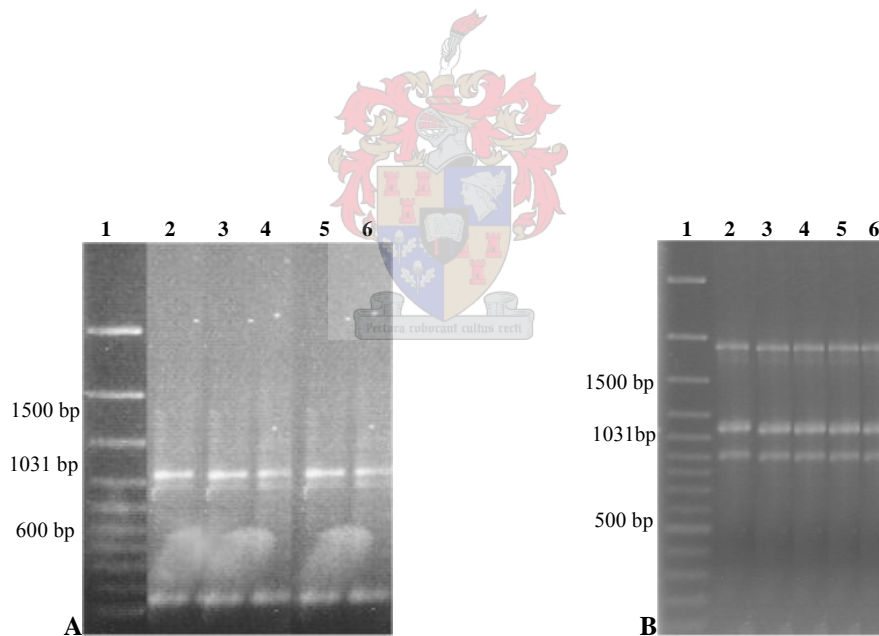


Figure 2. 26 Agarose gel electrophoresis of RAPD amplification products for the *Myroides* and *Empedobacter* spp isolates generated with primers P1 and P2, respectively. (A): Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 2-6: study isolates MY1, MY2, MY2B, MY3, MY3B, respectively. (B): Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); and lanes 2-6: study isolates MY1, MY2, MY2B, MY3, MY3B, respectively.

Eighteen RAPD fingerprints were produced for the 34 *Chryseobacterium/Elizabethkingia* spp. isolates and 6 reference strains with the P1 primer (Table 2.5; Figs. 2.27-2.29), while 20 fingerprints were obtained with the P2 primer (Table 2.5; Fig. 2.30-2.32). P1 fingerprints consisted of 3-9 fragments although 16 fragments were obtained for isolate CH34 (Table 2.5). Fingerprints ranged in size from approximately 164 bp to 4824 bp (Table 2.5).

Five common fragments of approximately 1900 bp, 1360 bp, 1212 bp, 965 bp and 895 bp were observed with primer P1 among RAPD profiles of different groups with the 1900 bp fragment being observed in 7 of the 18 groups, although the intensity did differ between the groups (Table 2.5; Figs. 2.27 – 2.29). No fragments were common to 100% of the study isolates. Reference strains were grouped separately into individual groups and did not cluster with any of the study isolates (Table 2.5; Figs. 2.27 – 2.29).

The 20 fingerprints generated by primer P2 consisted of 3-14 fragments ranging in size from approximately 157 bp to 3375 bp (Table 2.5). Nine fragments of approximately 2530 bp, 1965 bp, 1062 bp, 983 bp, 930 bp, 858 bp, 791 bp, 500 bp and 189 bp were observed for a number of subtypes (Table 2.5). A 500 bp fragment was observed in 7 out of the 20 groups, while a 189 bp fragment was observed in 6 subtypes, although the intensity did differ between the groups (Table 2.5; Figs. 2.30 – 2.32). No fragments were common to 100% of the study isolates. Subtypes RP2-1 and RP2-2 differed by the absence of a fragment of approximately 1407 bp present in the profiles of isolates in subtype RP2-2 (Table 2.5). Subtype RP2-3 shared this 1407 bp fragment with subtype RP2-1 but had five additional fragments of between 1407 bp and 744 bp in size (Table 2.5). Subtypes RP2-9 and RP2-10 differed by the absence of a 389 bp fragment present in subtype RP2-10 (Table 2.5). Additionally, subtypes RP2-9 and RP2-10 shared two fragments of approximately 500 bp and 189bp with subtypes RP2-1, RP2-2, and RP2-3 (Table 2.5).

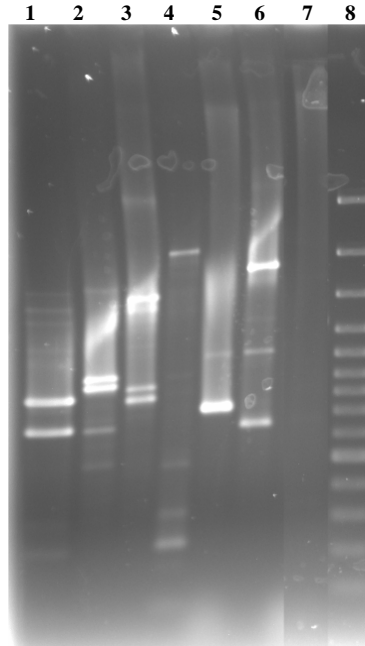
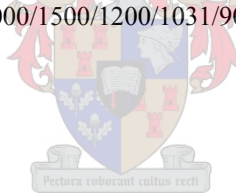


Figure 2.27 Agarose gel electrophoresis of RAPD amplification products for the reference strains generated with primer P1. Lanes 1-7: reference strains *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), *C. indoltheticum* (ATCC 27950), *C. joostei* (LMG 18212), and *E. meningoseptica* (NCTC 10016), *M. odoratus* (NCTC 11036), respectively; and lane 8: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada).



Although RAPD primer P1 clustered isolates CH1, CH1B and CH3 – CH9 into one group (RP1-1), RAPD primer P2 allowed for the differentiation of these isolates into two groups differentiating between isolates CH1 and CH1B, and isolates CH3 – CH9 (Table 2.5), respectively. As with PCR-RFLP typing, RAPD profiling indicated relatively high degree of genetic diversity among the *Chryseobacterium* and *Elizabethkingia* spp. isolates, and allowed for preliminary differentiation of these test isolates.

Table 2. 5 RAPD profiles of *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. study isolates and reference strains generated with primer P1.

Primer	Profiles				Isolates	No. of isolates
	No. of patterns	No. of fragments	Subtype	Size of fragments (kbp)		
P1	1 (<i>Myroides/Empedobacter</i>)	3	RP1-I	1151; 1043; 570	MY1, MY2, MY2B, MY3, MY3B	5
		0	RP1-0		<i>M. odoratus</i>	1
	18 (<i>Chryseobacterium/Elizabethkingia</i>)	4	RP1-1	1900; 1360; 992; 895	CH1, CH1B, CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9	10
		5	RP1-2	1900; 1760; 1607; 1360; 895	CH2, CH2B	2
		4	RP1-3	2100; 1484; 1102; 779	CH10	1
		4	RP1-4	1667; 1212; 958; 819	CH11, CH12	2
		4	RP1-5	3061; 2452; 1212; 1019	CH13, CH14	2
		7	RP1-6	3216; 2055; 1392; 1360; 1196; 965; 667	CH15, CH16, CH17, CH18, CH19	5
		5	RP1-7	1900; 1212; 743; 463; 348	CH21	1
		1	RP1-8	1227	CH22	1
		2	RP1-9	1900; 997	CH23, CH24	2
		3	RP1-10	1900; 1187; 849	CH25, CH26, CH27, CH28	4
		4	RP1-11	3694; 1900; 1188; 965	CH29, CH30, CH33	3
		16	RP1-12	4043; 2613; 2331; 2189; 1900; 1738; 1476; 1324; 1212; 1101; 895; 722; 639; 385; 284; 164	CH34	1
		9	RP1-13	1530; 1389; 1264; 1059; 962; 751; 613; 280; 200	<i>C. balustinum</i>	1
		5	RP1-14	1561; 882; 817; 625; 467	<i>C. gleum</i>	1
		6	RP1-15	3078; 1491; 1389; 1102; 841; 762	<i>C. indologenes</i>	1
		6	RP1-16	2031; 900; 829; 482; 318; 228	<i>C. indoltheticum</i>	1
3	RP1-17	4824; 1031; 710	<i>C. joostei</i>	1		
4	RP1-18	1851; 1314; 1059; 659	<i>E. meningoseptica</i>	1		

Table 2.5 (continued)

RAPD profiles of *Myroides* / *Empedobacter* spp. and *Chryseobacterium* / *Elizabethkingia* spp. study isolates and reference strains generated with primer P2.

Primer	Profiles				Isolates	No. of isolates
	No. of patterns	No. of fragments	Subtype	Size of fragments (kbp)		
P2	1 (<i>Myroides/Empedobacter</i>)	3	RP2-I	1889; 1110; 909	MY1, MY2, MY2B, MY3, MY3B	5
		5	RP2-II	2611; 1874; 858; 629; 481	<i>M. odoratus</i>	1
	20 (<i>Chryseobacterium/Elizabethkingia</i>)	5	RP2-1	2530; 1965; 1407; 500; 189	CH1, CH1B	2
		4	RP2-2	2530; 1965; 500; 189	CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9	8
		9	RP2-3	2.530; 1.407; 1.305; 1.181; 1.055; 0.810; 0.744; 0.500; 0.189	CH2, CH2B	2
		1	RP2-4	526	CH10	1
		3	RP2-5	1728; 1265; 695	CH11, CH12	2
		3	RP2-6	1407; 1140; 991	CH13, CH14	2
		3	RP2-7	1750; 707; 549	CH15, CH16, CH17, CH18, CH19	5
		3	RP2-8	1147; 930; 500	CH21	1
		5	RP2-9	1062; 930; 500; 389; 189	CH22	1
		3	RP2-10	1062; 500; 189	CH23, CH24	2
		6	RP2-11	1142; 1011; 894; 800; 500; 189	CH25, CH26, CH28	3
		6	RP2-12	2356; 2025; 1745; 983; 858; 791	CH27	1
		3	RP2-13	2768; 983; 791	CH29, CH30, CH33	3
		12	RP2-14	3375; 3156; 2453; 2199; 1200; 1129; 1052; 983; 858; 746; 620; 468	CH34	1
		3	RP2-15	1005; 496; 177	<i>C. balustinum</i>	1
		11	RP2-16	2493; 2341; 1406; 1316; 1248; 1031; 806; 684; 489; 248; 174	<i>C. gleum</i>	1
		10	RP2-17	2876; 2552; 1543; 1416; 967; 800; 653; 612; 537; 428	<i>C. indologenes</i>	1
		7	RP2-18	1987; 1273; 986; 744; 485; 418; 169	<i>C. indoltheticum</i>	1
7	RP2-19	2733; 1453; 1316; 1223; 512; 386; 157	<i>C. joostei</i>	1		
14	RP2-20	2454; 2015; 1481; 1361; 1290; 1018; 936; 879; 738; 616; 587; 469; 397; 163	<i>E. meningoseptica</i>	1		

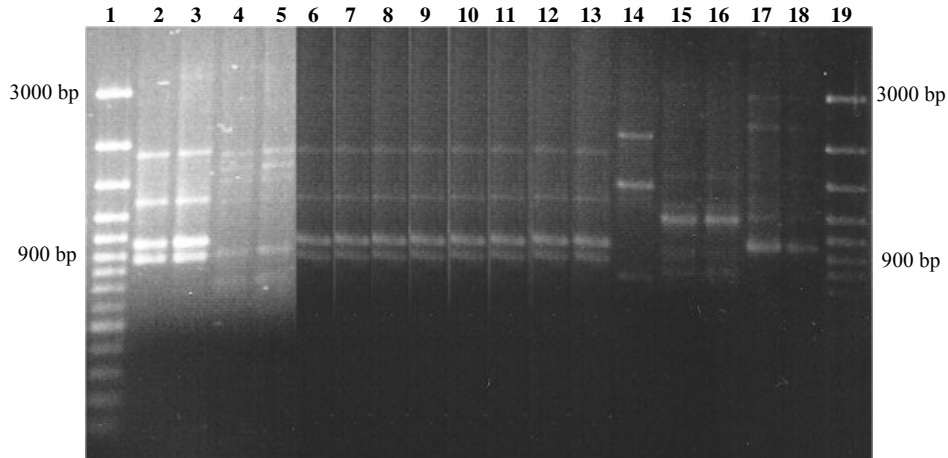


Figure 2. 27 Agarose gel electrophoresis of RAPD amplification products for the *Chryseobacterium* and *Elizabethkingia* spp. study isolates generated with primer P1. Lanes 1 and 19: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-5: isolates CH1, CH1B, CH2, and CH2B, respectively; lanes 6-13: isolates CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9, respectively; and lanes 14-18: isolates CH10, CH11, CH12, CH13 and CH14, respectively.

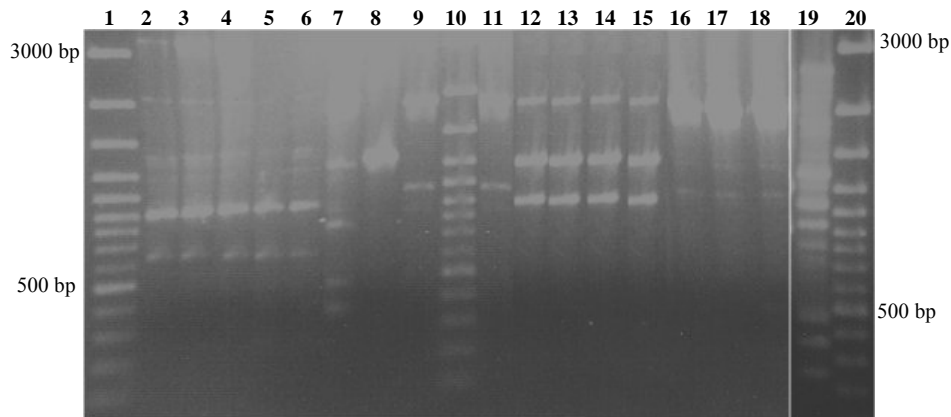
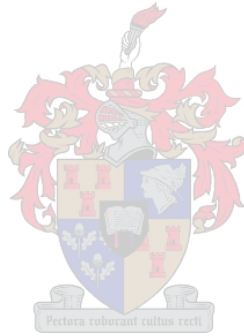


Figure 2. 28 Agarose gel electrophoresis of RAPD amplification products for the *Chryseobacterium* and *Elizabethkingia* spp isolates generated with primer P1. Lanes 1, 10 and 20: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-6: isolates CH15, CH16, CH17, CH18 and CH19, respectively; lanes 7-9: isolates CH21, CH22, and CH23, respectively; and lanes 11-19: isolates CH24, CH25, CH26, CH27, CH28, CH29, CH30, CH33 and CH34, respectively.

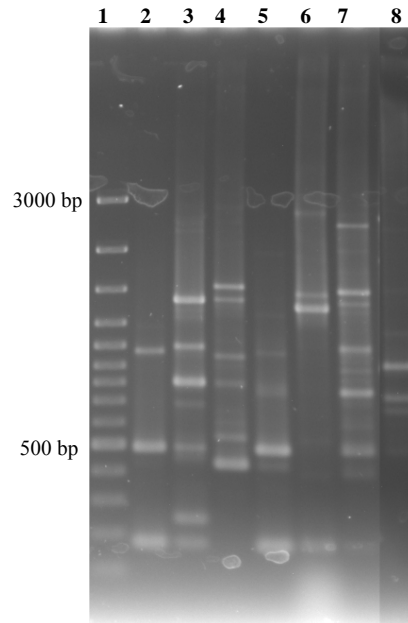


Figure 2. 29 Agarose gel electrophoresis of RAPD amplification products for the reference strains generated with primer P2. Lane 1: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-8: reference strains *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), *C. indoltheticum* (ATCC 27950), *C. joostei* (LMG 18212), *E. meningoseptica* (NCTC 10016), and *M. odoratus* (NCTC 11036), respectively.

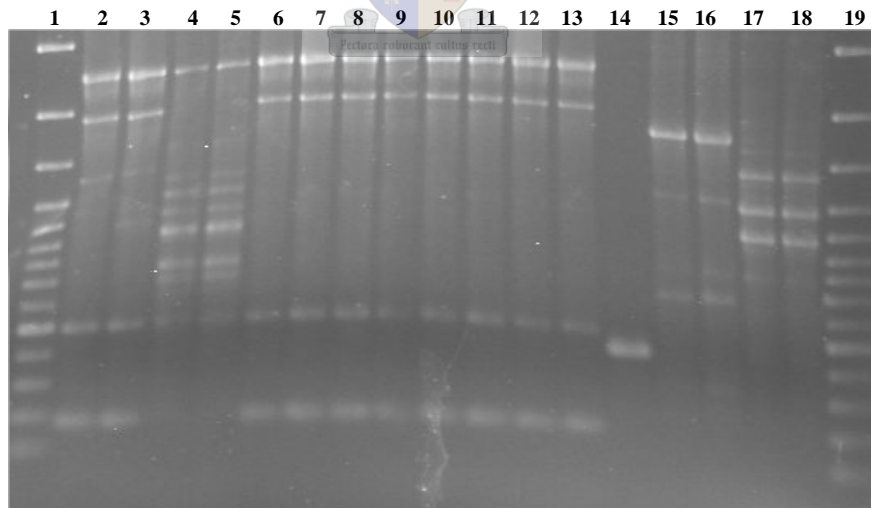


Figure 2. 30 Agarose gel electrophoresis of RAPD amplification products for the *Chryseobacterium* and *Elizabethkingia* spp. study isolates generated with primer P2. Lanes 1 and 19: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada), lanes 2-5: isolates CH1, CH1B, CH2, and CH2B, respectively, lanes 6-13: isolates CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9, respectively, and lanes 14-18: isolates CH10, CH11, CH12, CH13 and CH14, respectively.

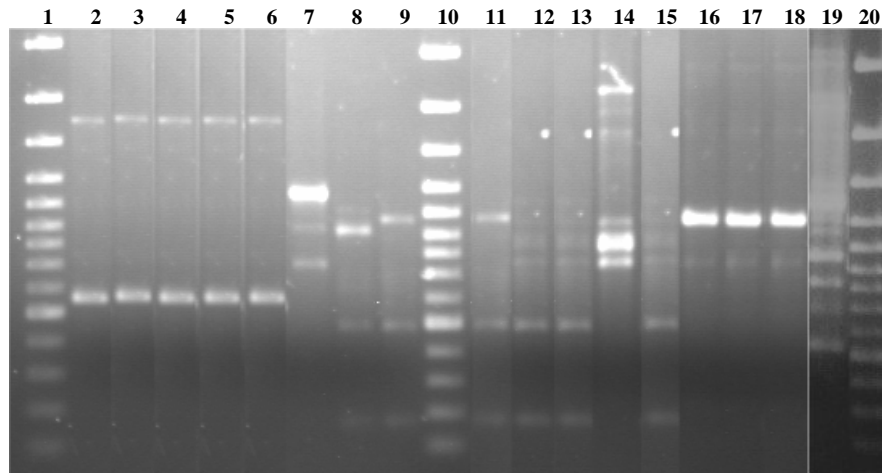


Figure 2.31 Agarose gel electrophoresis of RAPD amplification products for the *Chryseobacterium* and *Elizabethkingia* spp. study isolates generated with primer P2. Lanes 1, 10 and 20: O'GeneRuler 100 bp ladder plus (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada); lanes 2-6: isolates CH15, CH16, CH17, CH18 and CH19, respectively; lanes 7-9: isolates CH21, CH22, and CH23, respectively; and lanes 11-19: isolates CH24, CH25, CH26, CH27, CH28, CH29, CH30, CH33 and CH34, respectively.

Clusters obtained by Pearson product-moment correlation coefficient are shown in dendrograms (Figs. 2.33 – 2.36). With primer P1, a total number of 3 clusters were obtained for the *Myroides* and *Empedobacter* spp. isolates. These clusters displayed a maximum similarity level of 99.5%. The collection of isolates was divided into 2 major clusters (A and B) at a similarity level of approximately 93% (Fig. 2.33). Clusters A and B were further subdivided into 3 and 2 clusters, respectively, comprising isolates that had also been clustered visually. Although none of the isolates were clustered at 100% similarity, identical patterns were observed for all of the isolates during visual analysis (Table 2.5).

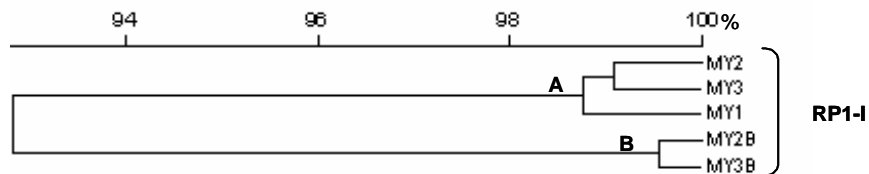


Figure 2.32 Dendrogram of the cluster analysis of RAPD profiles of *Myroides* and *Empedobacter* spp. isolates and reference strain following DNA amplification using primer P1.

With primer P1, a total number of 29 clusters were obtained for the *Chryseobacterium* and *Elizabethkingia* spp. isolates. These clusters displayed a maximum similarity level of 100%. The collection of isolates was divided into 2 major clusters (A and B) at a similarity level of approximately -14% (Fig. 2.34). Clusters A and B were further subdivided into 4 and 19 clusters, respectively, comprising isolates that had also been clustered visually. Cluster A contained all the reference strains included in this study, and cluster B comprised of the study isolates. Similarity levels of 100% were obtained for some of the isolates that were clustered together based on visual observations, i.e., CH2-CH2B (RP1-2), CH23-CH24 (RP1-9), CH25-CH28 (RP1-10), and CH29-CH33 (RP1-11). Study isolates clustered together in group RP1-1 were not clustered together with GelCompar analysis, although 100% similarity was calculated for some of the isolates belonging to this group (CH6-CH7, and CH3-CH4), similarity of 98% was observed between other isolates in this group (Fig. 2.34). Overall, all the *Chryseobacterium* and *Elizabethkingia* spp. isolates clustered at 63%, while the reference isolates clustered at 33%.

With primer P2, a total number of 5 clusters were obtained for the *Myroides* and *Empedobacter* spp. isolates. These clusters displayed a maximum similarity level of 99%. The collection of isolates was divided into 2 major clusters (A and B) at a similarity level of -29% (Fig. 2.35). Reference strain *M. odoratus* NTCC 11036, in cluster B was differentiated from the study isolates in cluster A which comprised of 4 clusters. Although none of the isolates were clustered at 100% similarity, identical patterns were observed for all of the isolates during visual analysis (Table 2.5). Due to differences in band intensities and gel alignment, clustering in GelCompar appeared to group isolates differently compared to visual clusters.

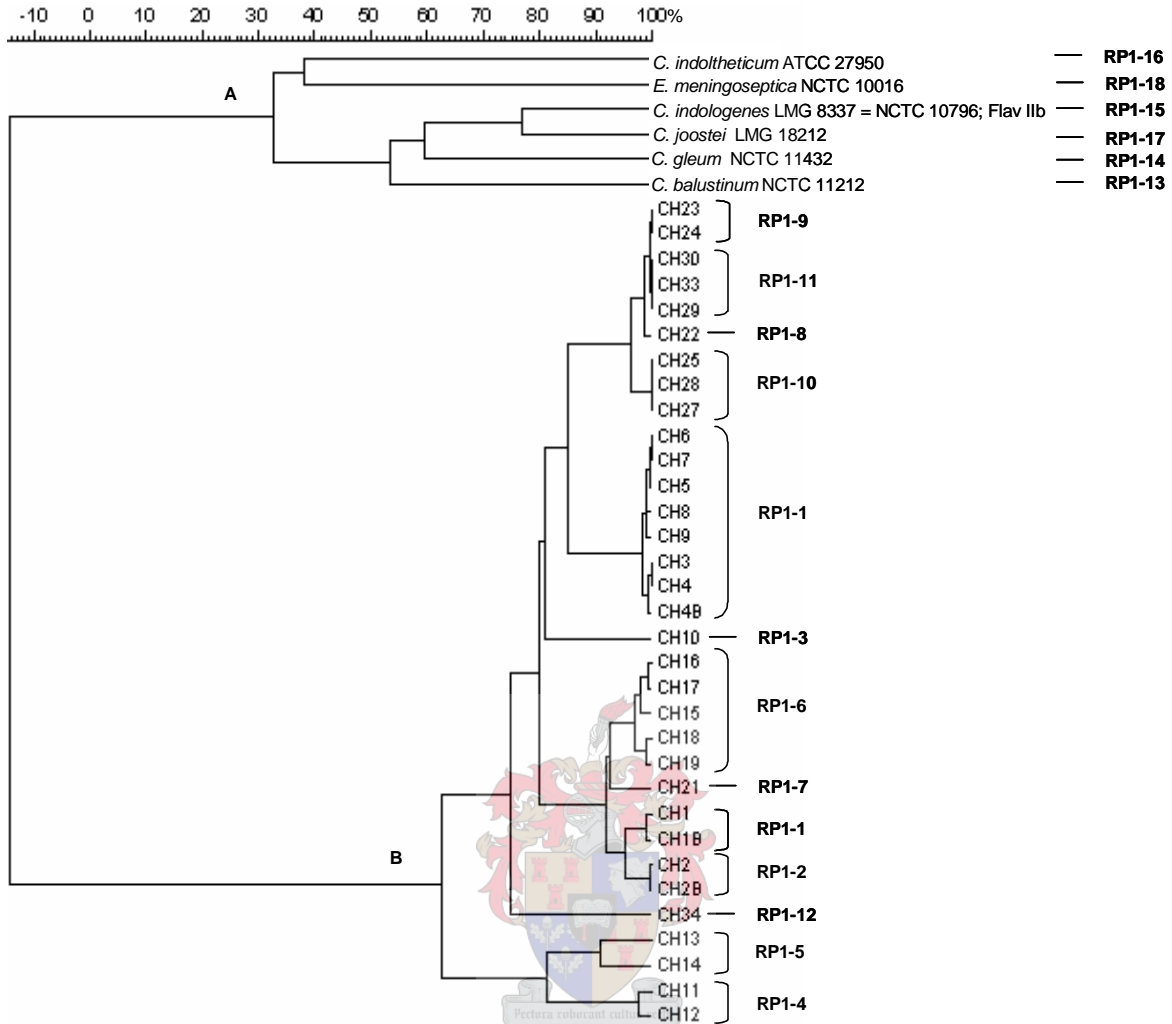


Figure 2. 33 Dendrogram of the cluster analysis of RAPD profiles of *Chryseobacterium/Elizabethkingia* spp. isolates and reference strains following DNA amplification using primer P1.

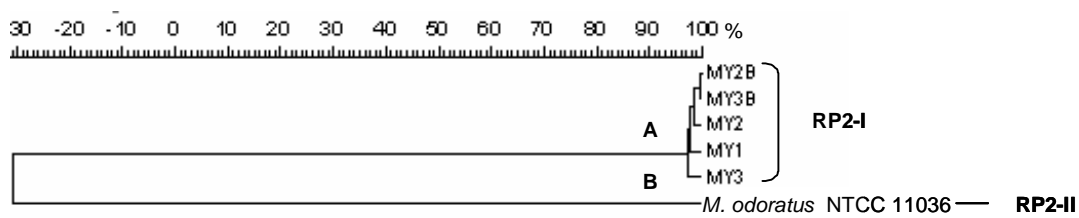


Figure 2. 34 Dendrogram of the cluster analysis of RAPD profiles of *Myroides* and *Empedobacter* spp. isolates and reference strain following DNA amplification using primer P2.

With primer P2, a total number of 36 clusters were obtained for the *Chryseobacterium* and *Elizabethkingia* spp. isolates. These clusters displayed a maximum similarity level of 100%. The collection of isolates was divided into 2 major clusters (A and B) at a similarity level of approximately 22% (Fig. 2.36). Clusters A and B were further subdivided into 16 and 18 clusters, respectively, comprising isolates that had also been clustered visually. Cluster B was further subdivided into clusters B1 and B2, cluster B1 contained all the reference strains included in this study, and displayed 63% similarity to cluster B2 which comprised of the study isolates. Similarity levels of 100% were obtained for some of the isolates that were clustered together based on visual observations, i.e., CH17 and CH19 (RP2-7), and CH29-CH30 (RP2-13). On the contrary, isolates sharing identical RAPD profiles showed different levels of similarity, i.e., CH1-CH1B (RP2-1) clustered at 98% similarity, CH3-CH9 (RP2-2) clustered at 99-95% similarity, CH2-CH2B (RP2-3) clustered at 99% similarity, CH11-CH12 (RP2-5) clustered at 98% similarity, CH13-CH14 (RP2-6) clustered at 98% similarity, CH15-CH19 (RP2-7) clustered at >99%, CH23-CH24 (RP2-10) clustered at 99% similarity, CH25-CH26 and CH28 (RP2-11) clustered at 97-99% similarity, respectively. Although CH29 and CH30 clustered at 100% similarity, CH33 included in group RP2-13 with these isolates only displayed 95% similarity to these isolates. Clustering in GelCompar appeared to group isolates differently compared to visual clusters, and may be ascribed to differences in band intensities and gel alignment.

2.3.5 Antimicrobial susceptibility tests

With respect to the aminoglycoside antibiotics, the *Myroides* and *Empedobacter* spp. study isolates displayed 100% resistance to amikacin, gentamicin and streptomycin (Table 2.6; Fig. 2.37). For the β -lactam antibiotics, isolates displayed 100% resistance to ampicillin, amoxicillin, augmentin, and oxacillin (Table 2.6; Fig. 2.37).

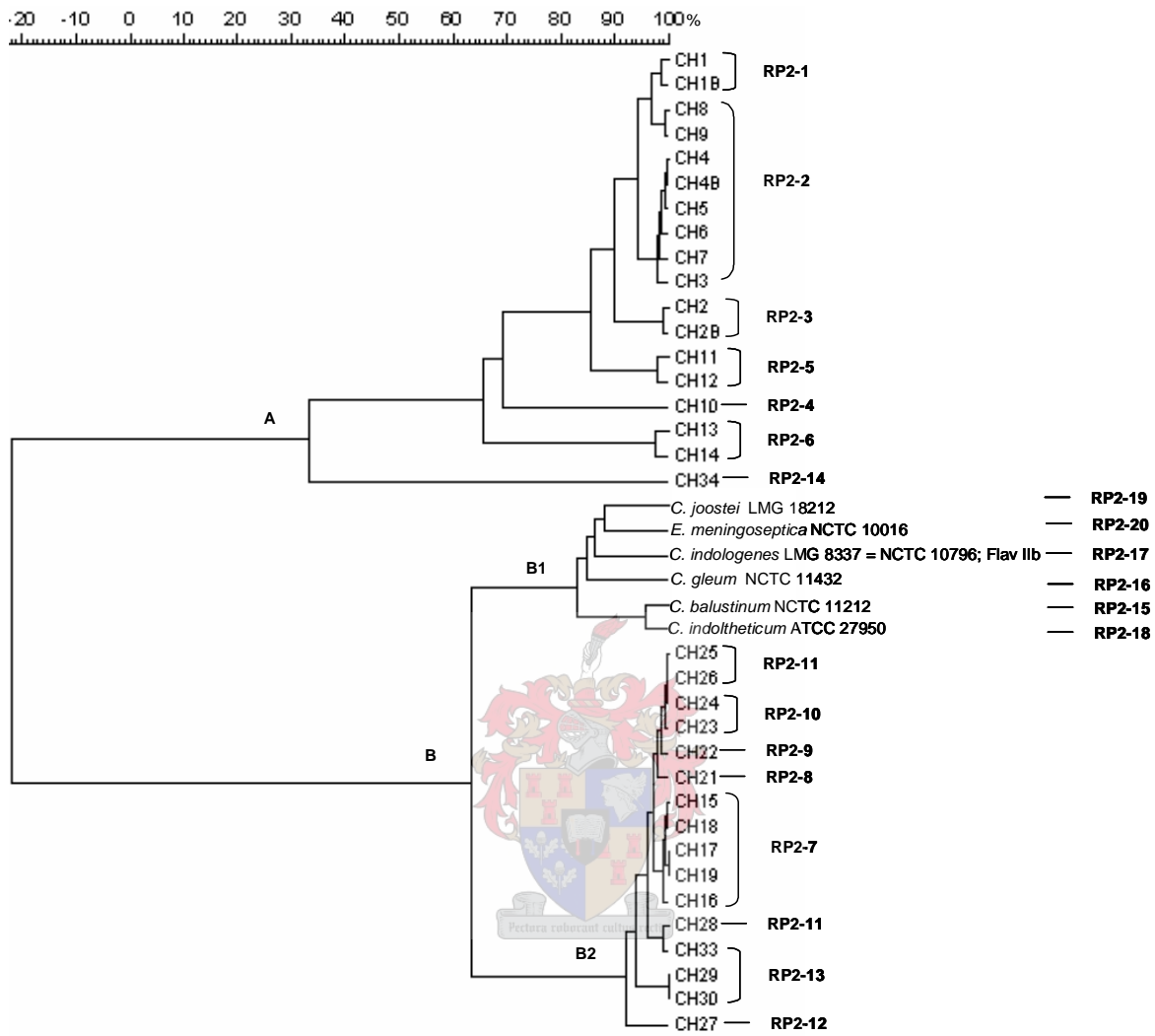


Figure 2. 35 Dendrogram of the cluster analysis of RAPD profiles of *Chryseobacterium/Elizabethkingia* spp. isolates and reference strains following DNA amplification using primer P2.

For the cephalosporins, isolates displayed 100% resistance to ceftriaxone and cefuroxime (Table 2.6; Fig. 2.37), while decreased susceptibility (40%) to ceftazidime and 100% susceptibility to cefoxitin were observed. Resistance to imipenem, a carbapenem, was observed for 80% of the study isolates, while that of piperacillin/tazobactam, an ureidopenicillin, was 60% (Table 2.6; Fig. 2.37). Study isolates displayed 100% susceptibility to azithromycin and only 40% erythromycin

susceptibility. Isolates displayed 100% resistance to tetracycline and 60% susceptibility to chloramphenicol, respectively (Table 2.6; Fig. 2.37). On comparison of quinolone zone diameters, 100% of the isolates were susceptible to nalidixic acid and norfloxacin but showed decreased susceptibility to ciprofloxacin (40%) and ofloxacin (80%), respectively (Table 2.6; Fig. 2.37). For the tetrahydrofolic acid biosynthesis inhibitors, 100% of the isolates were susceptible to sulphamethoxazole and cotrimoxazole but displayed 100% resistance to trimethoprim (Table 2.6; Fig. 2.37). MAR indices ranged between 0.48 – 0.56, and the most effective antibiotics against the *Myroides* and *Empedobacter* spp. isolates were azithromycin, ceftiofloxacin, cotrimoxazole, nalidixic acid, norfloxacin, and sulphamethoxazole.

Chryseobacterium and *Elizabethkingia* spp. study isolates displayed variation with respect to their antimicrobial susceptibility to the aminoglycoside antibiotics. For amikacin and streptomycin, majority of the isolates showed sensitivity with 44.12% and 58.82% susceptibility, respectively (Fig. 2.38). For lower concentrations of gentamicin (GM-10), 55.88% of the isolates were resistant, while 44.12% displayed intermediate resistance to higher concentrations of gentamicin (GM-120) (Table 2.6; Fig. 2.38). For the β -lactam antibiotics, majority of the study isolates displayed resistance to the penicillins - ampicillin (67.65%), amoxicillin (73.53%), augmentin (61.76%), and oxacillin (91.18%) (Table 2.6; Fig. 2.38). Susceptibility (64.71%) to the carbapenem, imipenem, was observed, while 100% of isolates were susceptible to piperacillin-tazobactam, an ureidopenicillin (Table 2.6; Fig. 2.38). Majority of the isolates were susceptible to the macrolide antibiotics, azithromycin (50%) and erythromycin (44.12%), respectively, while 52.94% percent of the isolates displayed resistance to chloramphenicol but 41.18% were susceptible to tetracycline (Table 2.6; Fig. 2.38). Resistance to the folic acid biosynthesis inhibitor, sulphamethoxazole, was observed for 82.35% of the study isolates, while only 11.76% and 5.88% resistance, respectively, was observed for trimethoprim and cotrimoxazole (Table 2.6; Fig. 2.38). High levels of susceptibility was observed for the quinolone antibiotics including ciprofloxacin (61.76%), ofloxacin (73.53), nalidixic acid (73.53%), and norfloxacin (76.47%) (Table 2.6; Fig. 2.38). MAR indices ranged from 0.04 to 0.64 for the *Chryseobacterium/Elizabethkingia* spp. study isolates. Twenty-four (70.6%) of the study isolates displayed

MAR indices higher than 0.2, and eight (23.5%) of the isolates displayed MAR indices higher than 0.5 (Table 2.6; Fig. 2.38).

The most effective antibiotics against the *Chryseobacterium* and *Elizabethkingia* spp. isolates were piperacillin-tazobactam, ceftazidime, ceftaxone, imipenem and the quinolone antibiotics (Table 2.6; Fig. 2.38).

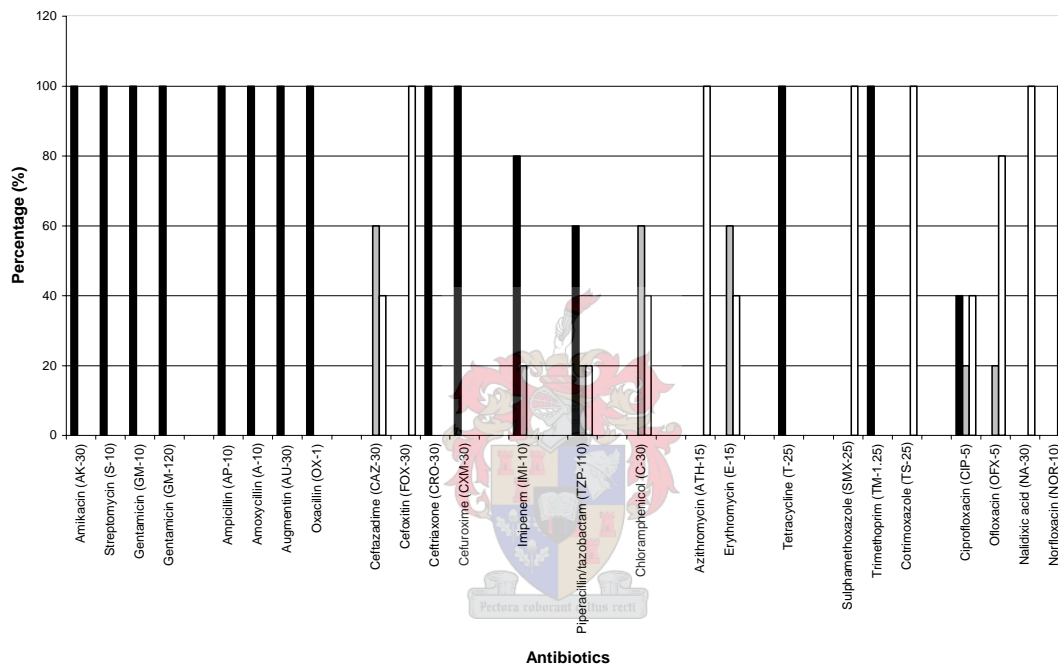


Figure 2.36 Percentages of resistance (■), intermediate susceptibility (▒), and susceptibility (□) of the *Myroides* and *Empedobacter* spp. isolates to the panel of antibiotics tested.

Table 2. 6 Antibigrams for *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates.

Antibiotic	<i>Myroides / Empedobacter</i> spp.					<i>Chryseobacterium / Elizabethkingia</i> spp. isolates															
	MY1	MY2	MY2B	MY3	MY3B	CH1	CH1B	CH2	CH2B	CH3	CH4	CH4B	CH5	CH6	CH7	CH8	CH9	CH10	CH11	CH12	CH13
Amikacin (AK-30)	R	R	R	R	R	R	R	R	R	I	R	I	S	S	S	S	R	I	I	S	R
Ampicillin (AP-10)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	S
Amoxicillin (A-10)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	S
Augmentin (AU-30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	S
Azithromycin (ATH-15)	S	S	S	S	S	R	R	R	S	R	R	R	R	R	R	R	S	S	S	S	S
Ceftazadime (CAZ-30)	S	S	I	I	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin (FOX-30)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S
Ceftriaxone (CRO-30)	R	R	R	R	R	I	I	I	R	S	I	I	S	S	S	S	S	S	R	I	S
Cefuroxime (CXM-30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R	S
Chloramphenicol (C-30)	I	I	S	I	S	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S
Ciprofloxacin (CIP-5)	R	I	S	R	S	R	R	I	R	R	I	R	S	S	S	S	S	S	S	S	S
Cotrimoxazole (TS-25)	S	S	S	S	S	I	I	S	I	S	S	S	S	S	S	S	S	S	I	S	S
Erythromycin (E-15)	I	I	S	I	S	R	R	R	I	R	R	R	R	R	R	R	S	S	I	S	S
Gentamicin (GM-10)	R	R	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	S	S	S	R
Gentamicin (GM-120)	R	R	R	R	R	I	I	I	R	I	I	I	S	S	I	I	I	I	S	S	I
Imipenem (IMI-10)	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S
Nalidixic acid (NA-30)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
Norfloxacin (NOR-10)	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
Ofloxacin (OFX-5)	I	S	S	S	S	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
Oxacillin (OX-1)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Piperacillin/tazobactam (TZP-110)	S	S	R	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole (SMX-25)	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	I	S	R	R	S
Streptomycin (S-10)	R	R	R	R	R	S	S	S	S	S	S	S	S	S	I	I	I	S	I	S	R
Tetracycline (T-25)	R	R	R	R	R	I	R	R	R	R	I	R	S	S	R	R	S	S	I	S	S
Trimethoprim (TM-1.25)	R	R	R	R	R	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S
MAR indices	0.56	0.52	0.56	0.56	0.48	0.56	0.56	0.56	0.64	0.56	0.52	0.56	0.44	0.44	0.48	0.48	0.16	0.04	0.28	0.24	0.20

R – Resistant
I – Intermediate susceptibility
S - Susceptible

Table 2.6 (Continued). Antibigrams for *Chryseobacterium* and *Elizabethkingia* spp. isolates.

Antibiotic	<i>Chryseobacterium</i> / <i>Elizabethkingia</i> spp. isolates																	
	CH14	CH15	CH16	CH17	CH18	CH19	CH21	CH22	CH23	CH24	CH25	CH26	CH27	CH28	CH29	CH30	CH33	CH34
Amikacin (AK-30)	R	R	I	R	R	R	S	S	S	S	S	S	S	S	S	R	S	R
Ampicillin (AP-10)	S	R	R	R	R	R	R	S	S	S	R	S	R	S	R	S	S	R
Amoxycillin (A-10)	R	R	R	R	R	R	R	S	S	S	R	I	R	S	R	I	S	R
Augmentin (AU-30)	S	R	R	R	R	R	S	S	S	S	I	S	R	S	R	S	S	R
Azithromycin (ATH-15)	S	I	I	I	I	S	S	S	S	S	S	S	S	S	I	S	S	R
Ceftazadime (CAZ-30)	S	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	R	S
Cefoxitin (FOX-30)	S	S	I	R	I	S	S	S	S	S	S	S	R	S	S	S	S	S
Ceftriaxone (CRO-30)	S	R	R	I	R	I	S	S	S	S	S	S	S	S	S	S	S	I
Cefuroxime (CXM-30)	S	R	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S	R
Chloramphenicol (C-30)	S	R	R	I	R	R	S	S	S	S	S	S	R	S	I	R	S	R
Ciprofloxacin (CIP-5)	S	I	I	I	S	I	S	S	S	S	I	S	S	S	S	S	S	I
Cotrimoxazole (TS-25)	S	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	S	I
Erythromycin (E-15)	S	I	I	S	I	I	S	S	S	S	S	S	R	S	I	S	S	R
Gentamicin (GM-10)	R	R	R	I	R	R	S	S	S	S	S	S	S	S	S	R	S	R
Gentamicin (GM-120)	R	R	R	R	R	I	S	S	S	S	S	S	S	I	I	S	R	I
Imipenem (IMI-10)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
Nalidixic acid (NA-30)	I	S	S	S	S	S	S	S	R	R	R	R	S	R	S	R	R	S
Norfloxacin (NOR-10)	S	S	S	S	S	S	S	S	S	I	R	I	S	I	I	S	I	I
Ofloxacin (OFX-5)	S	S	R	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S
Oxacillin (OX-1)	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	R	R
Piperacillin/tazobactam (TZP-110)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole (SMX-25)	R	S	R	R	R	R	R	I	R	R	R	R	R	R	R	S	R	R
Streptomycin (S-10)	R	R	R	R	R	R	S	S	S	S	S	S	S	S	R	R	S	R
Tetracycline (T-25)	S	I	I	R	I	R	R	R	S	S	S	S	S	S	I	R	S	R
Trimethoprim (TM-1.25)	S	S	S	S	S	S	S	S	I	I	R	I	I	I	S	R	R	R
MAR indices	0.28	0.44	0.48	0.44	0.48	0.44	0.20	0.08	0.20	0.08	0.24	0.12	0.40	0.12	0.28	0.28	0.24	0.60

R – Resistant
 I – Intermediate susceptibility
 S - Susceptible

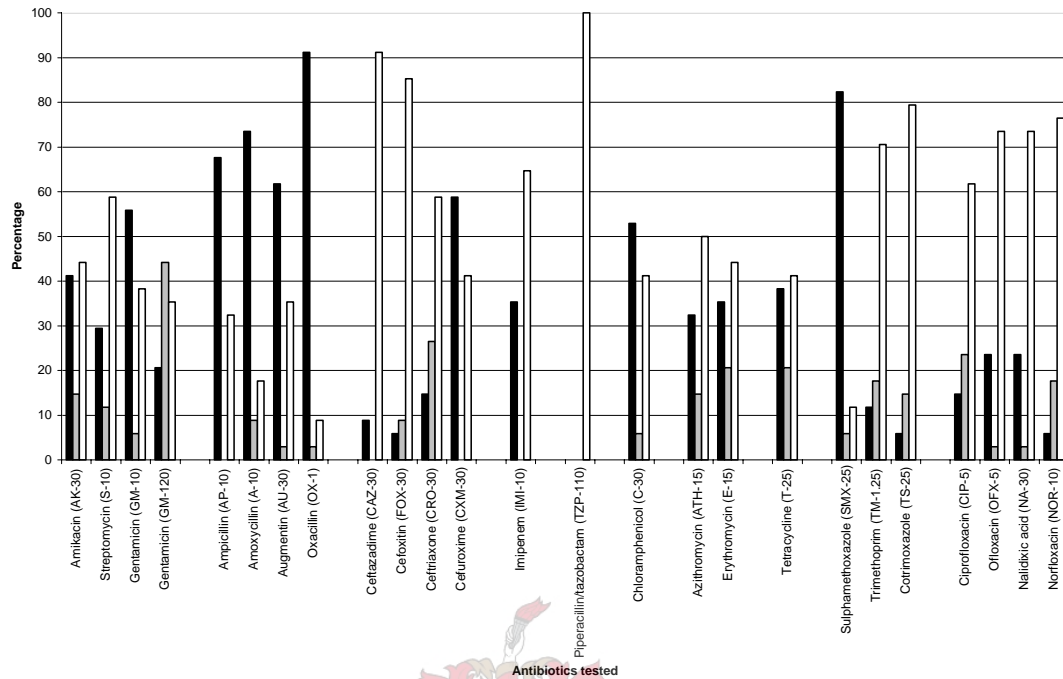


Figure 2.37 Percentages of resistance (■), intermediate susceptibility (▒), and susceptibility (□) of the *Chryseobacterium* and *Elizabethkingia* spp. isolates to the panel of antibiotics tested.

2.3.6 Whole Cell Protein (WCP) analysis

Analysis of WCPs identified a single WCP profile for all five *Myroides* and *Empedobacter* spp. isolates, consisting of 19 protein bands ranging in size from 40 kDa to 17 kDa (Table 2.7). No differentiation could be made between the study isolates (Fig. 2.39) or between study isolates and the reference strain *M. odoratus* (NTCC 11036) (Fig. 2.39).

Fifteen WCP profiles were obtained for the *Chryseobacterium* and *Elizabethkingia* spp. study isolates and reference strains (Table 2.7; Figs. 2.40 – 2.43). Profiles consisted of 12 to 35 protein fragments ranging in size from 10 kDa to 255 kDa (Table 2.7; Figs. Figs. 2.40 – 2.43). Reference strains were grouped separately into individual groups and could not be clustered with any of the study isolates (Table 2.7; Figs. 2.40 – 2.43), as was observed with RAPD analysis.

Thirteen protein bands ranging in size from 16 kDa to 109 kDa were common to *Chryseobacterium* and *Elizabethkingia* spp. isolates WCP profiles, with 47% of the isolates displaying bands of 86 kDa, 60 kDa, and 46 kDa, and 53% displaying bands of 109 kDa, 69 kDa, 58 kDa, and, 16 kDa in size. With the exception of isolates CH23 and CH24 (Table 2.7; Figs. 2.40 – 2.43), a 50 kDa protein band was common to all the study isolates (Table 2.7; Figs. 2.40 – 2.43), while 60% of the isolates displayed protein bands of 55 kDa and 41 kDa. All the reference strains displayed a protein band of 18 kDa, and 67% of the isolates (including 4 reference strains) possessed a 32 kDa protein (Table 2.7; Fig 2.40 – 2.43). Although all of the study isolates possessed a ~36 kDa protein, no protein bands were common to all of the study isolates and reference strains.

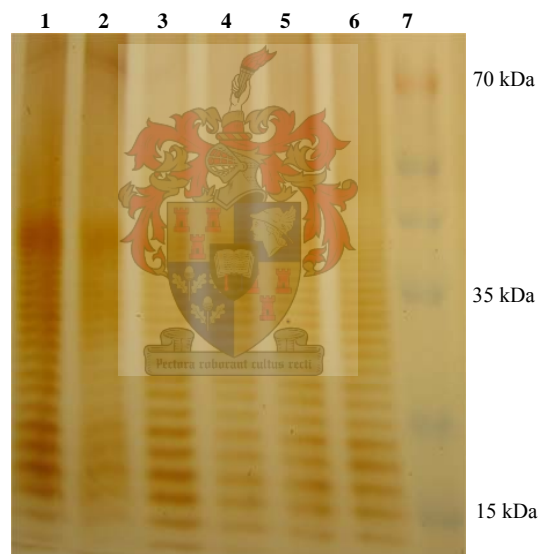


Figure 2.39 Electrogram displaying whole cell protein profiles of *Myroides* and *Empedobacter* spp. isolates. Lanes 1-6: isolates *M. odoratus* (NTCC 11036), MY1, MY2, MY2B, MY3, MY3B, respectively; and lane 7: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada).

Table 2.7 WCP profiles for *Myroides* and *Empedobacter* and *Chryseobacterium* and *Elizabethkingia* spp. study isolates and reference strains.

Profiles				Isolates	No. of isolates
No. of patterns	Subtype	No. of fragments	Size of fragments (KDa)		
1 (<i>Myroides/ Empedobacter</i>)	W-1	19	40; 39; 38; 37; 36; 35; 34; 33; 32; 31; 30; 29; 28; 27; 26; 25; 23; 21; 17	MY1, MY2, MY2B, MY3, MY3B, <i>M. odoratus</i>	6
15 (<i>Chryseobacterium/ Elizabethkingia</i>)	W-1	30	189; 163; 157; 141; 130; 124; 116 ; 109; 101 ; 93; 89; 86; 83; 79; 69; 66; 63; 62; 58; 55 ; 51 ; 46 ; 41; 39; 38; 36; 35 ; 25 ; 21 ; 18	CH1, CH1B, CH3, CH4, CH4B, CH5, CH6, CH7, CH8, CH9, CH21, CH29, CH30, CH33, CH34	15
	W-2	13	189; 168; 116 ; 101; 91; 73; 62; 60; 51 ; 44 ; 36; 32; 13	CH2, CH2B	2
	W-3	19	169; 160; 133; 119; 115; 110; 98 ; 95; 84; 79; 73; 70; 67; 65; 56; 50 ; 37; 36 ; 33	CH10, CH15, CH16, CH17, CH18, CH19	6
	W-4	35	255; 226; 217; 188; 183; 177; 160; 154; 141; 134; 128; 125; 122; 116; 110 ; 101; 93; 81; 77; 75; 69; 66; 62; 59; 55; 50 ; 47; 39; 37; 35; 31; 27; 17; 16; 15	CH11, CH12	2
	W-5	25	232; 226; 221; 181; 170; 148; 143; 121; 115; 112; 106; 104; 97; 87; 82; 69; 67; 59; 52; 47 ; 37; 35; 32; 28; 16	CH13, CH14	2
	W-6	26	189; 163; 154; 128; 126; 124; 116; 114; 110; 105; 95; 89; 80; 73; 70; 67; 65; 62; 60; 59; 50 ; 43; 40; 37; 35; 34	CH22	1
	W-7	14	183; 153; 138; 128; 121; 112; 98; 82; 68; 64; 61; 59; 53; 47	CH23, CH24	2
	W-8	35	218 ; 189; 174; 153 ; 140; 130; 128 ; 118 ; 112; 109; 106; 103; 90 ; 85; 75; 69; 67; 66; 64; 60; 57; 55; 51; 41 ; 38; 37; 35 ; 31; 29; 27; 22; 17; 16; 15; 13	CH25, CH26, CH28	3
	W-9	28	212; 206; 200; 178; 162 ; 143; 135; 129; 118; 113 ; 107; 97; 87 ; 73; 68 ; 59; 56 ; 52 ; 47 ; 42; 38 ; 36; 34 ; 33; 30; 29 ; 24; 19	CH27	1
	W-10	12	126; 112; 99 ; 87; 82; 60; 50; 41; 40; 23 ; 19; 16	<i>C. balustinum</i>	1
	W-11	23	217; 205; 187; 173; 131; 112; 96 ; 84; 72; 59; 54; 47; 43 ; 40 ; 37 ; 35; 34; 31; 24 ; 18 ; 15 ; 13; 11	<i>C. gleum</i>	1
	W-12	28	170; 141; 119; 110; 106; 101; 94; 89; 82; 79; 71; 54; 45; 40; 39; 37 ; 34; 31; 29; 25; 23; 21; 19 ; 17; 13; 12; 11; 10	<i>C. indologenes</i>	1
	W-13	16	114; 109; 92; 86; 73; 40 ; 39 ; 38 ; 37 ; 25 ; 18; 16; 14; 12 ; 11; 10	<i>C. joostei</i>	1
	W-14	25	196; 175; 162; 149; 136; 114; 107; 95; 89; 86; 77; 55; 43; 40 ; 39; 38 ; 37; 34; 32; 26; 25; 20 ; 17; 14; 11	<i>C. indoltheticum</i>	1
	W-15	22	198; 172; 109 ; 102 ; 91 ; 81 ; 69; 62; 56; 47; 39 ; 38 ; 35 ; 33 ; 28; 25; 20; 17; 15; 13; 11; 10	<i>E. meningoseptica</i>	1

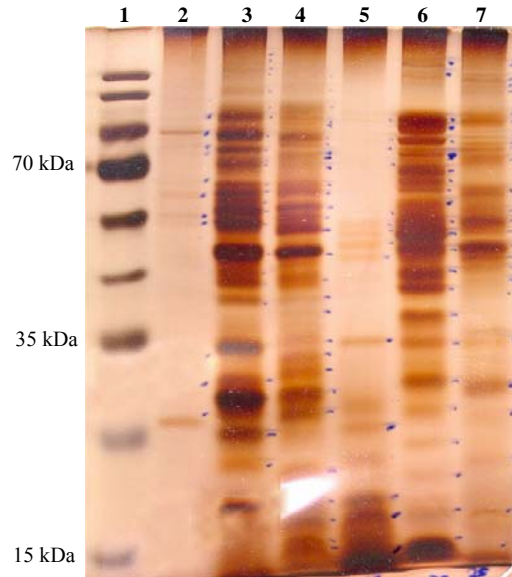


Figure 2. 38 Electrogram displaying whole cell protein profiles of *Chryseobacterium* and *Elizabethkingia* reference strains. Lane 1: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada); and lanes 2-7: reference strains *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), *C. joostei* (LMG 18212), *E. meningoseptica* (NCTC 10016), and *C. indoltheticum* (ATCC 27950), respectively.

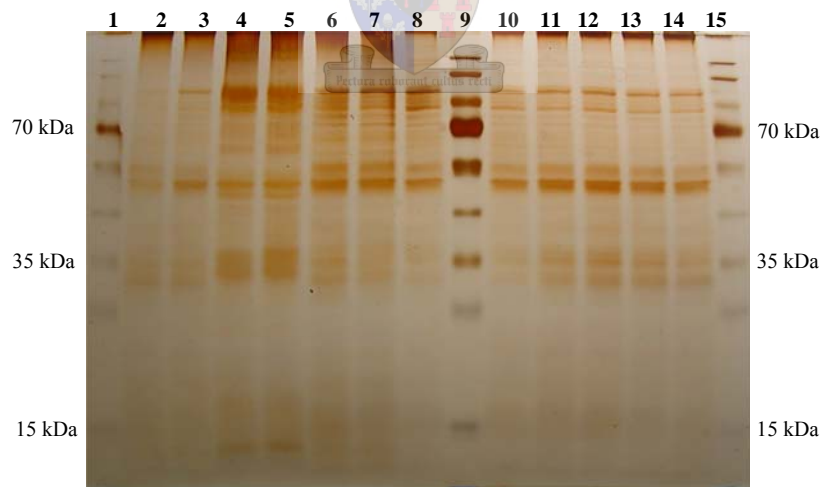


Figure 2. 39 Electrogram displaying whole cell protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lanes 1, 9, and 15: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada); lanes 2-8: study isolates CH1, CH1B, CH2, CH2B, CH3, CH4, CH4B; and lanes 10-14: CH5, CH6, CH7, CH8, CH9, and respectively.

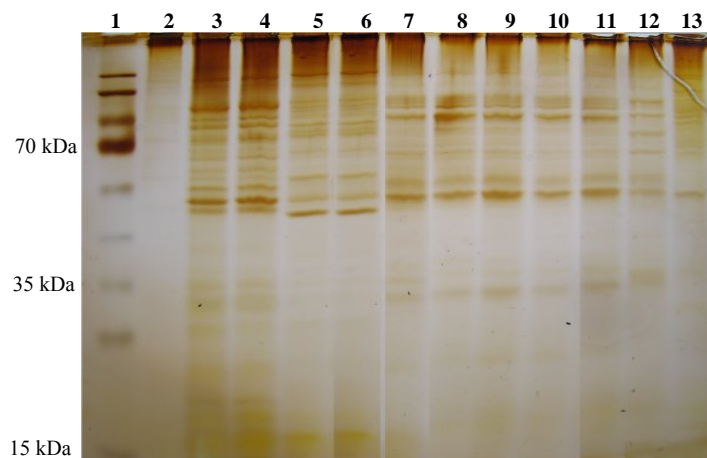


Figure 2. 40 Electrogram displaying whole cell protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lane 1: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada); and lanes 2-13: CH10, CH11, CH12, CH13, CH14, CH15, CH16, CH17, CH18, CH19, and CH21, respectively.

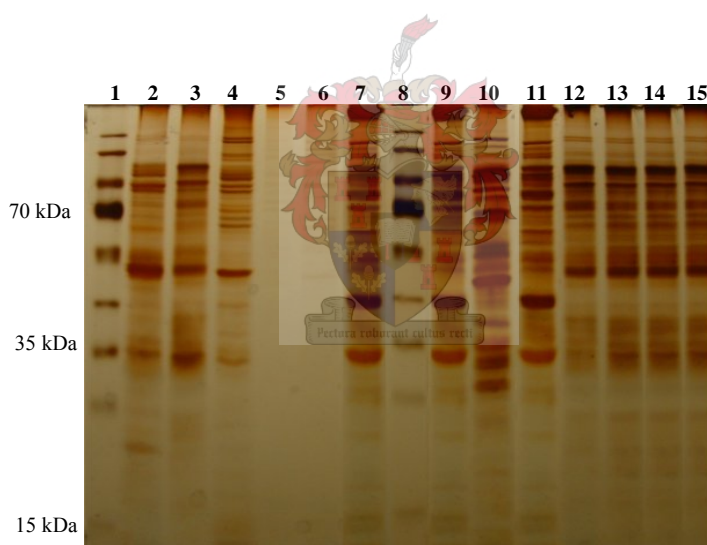


Figure 2. 41 Electrogram displaying whole cell protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lanes 1 and 8: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada); lanes 2-7: CH19, CH21, CH22, CH23, CH24, CH25; and lanes 9-15: CH26, CH27, CH28, CH29, CH30, CH33, and CH34, respectively.

Clusters obtained by the Pearson product-moment correlation coefficient are shown in dendograms (Figs. 2.44-2.45). For the *Myroides* and *Empedobacter* spp. isolates a total of 5 clusters were established at a maximum similarity level of 92% (Fig.

2.44). The collection of isolates was divided into 2 major clusters (A and B) at 55% similarity. Cluster A contained study isolate MY1 and the reference strain *M. odoratus* NTCC 11036 displaying 68% similarity. Cluster B contained two distinct branches which displayed 76% similarity, with MY2 and MY2B on one branch displaying 89% similarity, and MY3 and MY3B on the other branch displaying 92% similarity. Although none of the isolates were clustered at 100% similarity, identical profiles were observed during visual analysis for both the study isolates and the reference strain (Table 2.7). These differences may be ascribed to differences in protein band intensities and gel alignment, which leads to different clustering by GelCompar analysis compared to visual clustering.

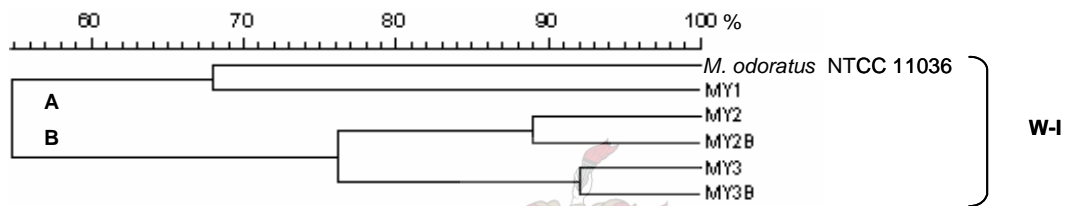


Figure 2. 42 Dendrogram generated by cluster analysis of whole cell protein profiles of the *Myroides/ Epedobacter* spp. isolates and reference strain.

For the *Chryseobacterium* and *Elizabethkingia* spp. isolates a total of 37 clusters were established at a maximum similarity level of 100% (Fig. 2.45). The collection of isolates was divided into 2 major clusters (A and B) at 14% similarity. Cluster A contained three distinct branches (A1-3), branch A1 containing 5 of the reference strains displayed 35% similarity with A2 containing study isolates only, and branch A3 containing only reference strain *C. balustinum* NCTC 11212 displayed 27% similarity to branches A2 and A3. Cluster B contained two distinct branches comprised of study isolates only which displayed 45% similarity. Groups W-2, W-4, W-5, W-7 and W-8, respectively, contained isolates displaying identical protein profiles during visual analysis, and displayed similarity $\geq 97\%$ between isolates in the respective groups (Table 2.7; Fig. 2.45). Isolates grouped together in group W-3, were separated into the two branches of cluster B with isolates CH3 and CH4 in one branch and majority of these isolates (CH1-CH1B, CH5-CH9, and CH21) in the other branch. Isolates belonging to group W-1 were separated by the two major clusters, as well as by the two branches in

cluster B. Although isolates belonging to groups W-1 to W-8 appeared identical during visual analysis, 100% similarity was not observed by GelCompar analysis. These differences may be attributed to differences in protein band intensities and gel alignment.

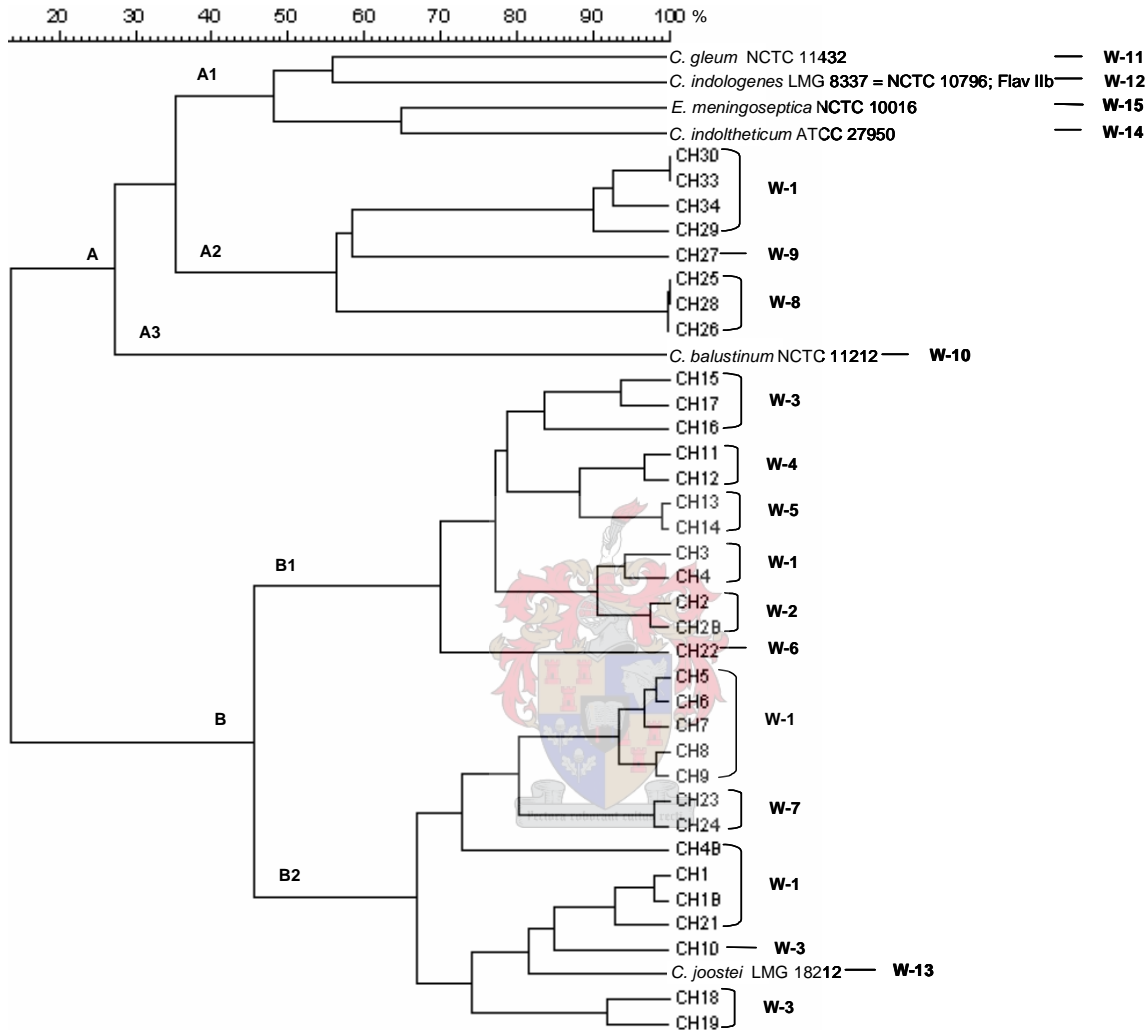


Figure 2. 43 Dendrogram generated by cluster analysis of whole cell protein profiles of the *Chryseobacterium* and *Elizabethkingia* spp. isolates and reference strains.

2.3.6 Outer Membrane Protein (OMP) analysis

Two different profiles were established following visual analysis of OMP profiles of the *Myroides* and *Empedobacter* spp. isolates (Table 2.8; Fig. 2.46). These profiles consisted of 10 and 11 bands respectively, ranging in size from approximately 272 kDa to

31 kDa. Isolates were grouped as follows: isolates MY1, MY3 (O-I), and isolates MY2, MY2B, MY3B and *M. odoratus* (NTCC 11036) (O-II), respectively (Table 2.8). Profiles differed by the presence/absence of one OMP band of approximately 34 kDa (Table 2.8). Two over-expressed OMPs, of approximately 38 kDa and 31 kDa were observed in both OMP profiles.

Twenty-two different OMP profiles were observed for the *Chryseobacterium* and *Elizabethkingia* spp. isolates (Table 2.8; Figs. 2.47 - 2.52). OMP profiles consisted of 2 - 19 fragments, ranging in size from approximately 200 kDa to 18 kDa (Table 2.8; Figs. 2.47 - 2.52). Six fragments of approximately 60 kDa, 48 kDa, 32 kDa, 30 kDa, 24 kDa, and 21 kDa were observed among different subtypes of isolates. The 60 kDa, 48 kDa, 30 kDa fragments were common to 50% of the isolates, while a 24 kDa fragment was observed for 48% of the isolates. A 32 kDa and 21 kDa fragment, respectively, were observed for 62% and 67% of the isolates (Table 2.8; Figs. 2.47 - 2.52). The intensity of the fragments, however, differed among the different fragments (Table 2.8; Figs. 2.47 - 2.52). No fragments were common to all of the test isolates. OMP profiles obtained for the study isolates did not correspond to those obtained for the reference strains (Figs. 2.47 - 2.52), but reference strains *C. joostei* (LMG 18212) and *C. indoltheticum* (ATCC 27950) shared identical profiles (Table 2.8; Fig. 2.48).

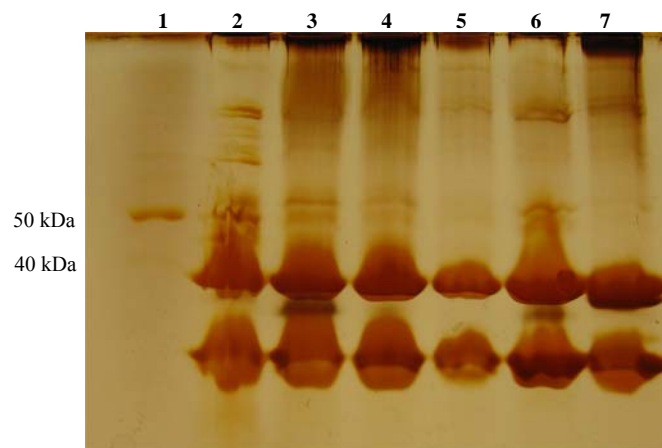


Figure 2. 44 Electroblotting displaying outer membrane protein profiles of *Myroides* and *Empedobacter* spp. isolates. Lane 1: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada); and lanes 2-7: isolates *M. odoratus* (NTCC 11036), MY1, MY2, MY2B, MY3, and MY3B, respectively.

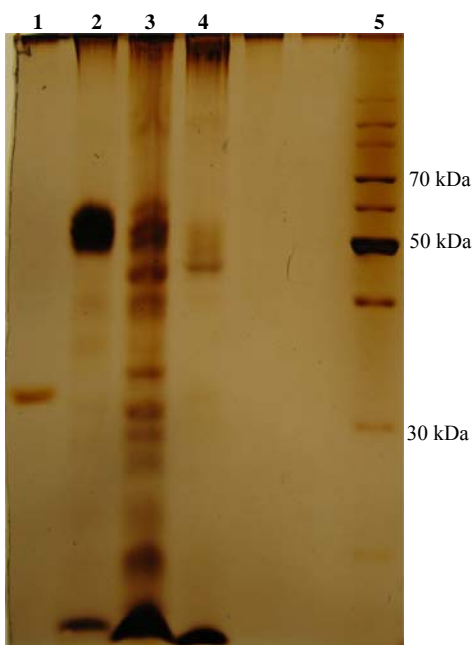


Figure 2. 45 Electrogram displaying outer membrane protein profiles of *Chryseobacterium* and *Elizabethkingia* reference strains. Lanes 1-4: reference strains *C. balustinum* (NCTC 11212), *C. gleum* (NCTC 11432), *C. indologenes* (LMG 8337 = NCTC 10796; Flav IIb), and *E. meningoseptica* (NCTC 10016), respectively; and lane 5: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada).

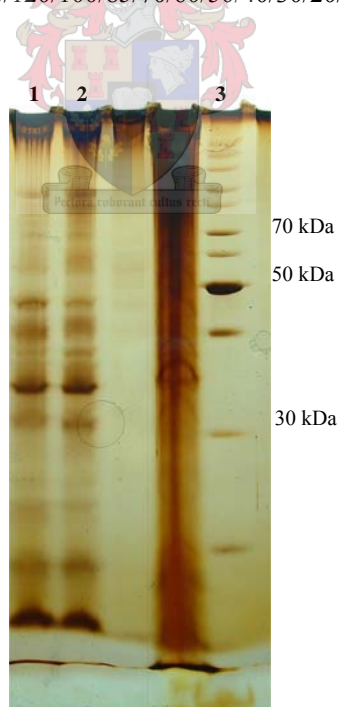


Figure 2. 46 Electrogram displaying outer membrane protein profiles of *Chryseobacterium* and *Elizabethkingia* reference strains. Lanes 1 and 2: reference strains *C. joostei* (LMG 18212), *C. indoltheticum* (ATCC 27950), respectively; and lane 3: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada).

Table 2. 8 OMP profiles of *Myroides* and *Empedobacter* and *Chryseobacterium* and *Elizabethkingia* spp isolates and reference strains.

No. of patterns	Profiles			Isolates	No. of isolates
	Subtype	No. of fragments	Size of fragments (Kbp)		
2 (<i>Myroides</i> / <i>Empedobacter</i>)	O-I	11	272; 112; 106; 92; 86; 78; 75; 72; 38 ; 34; 31	MY1, MY3	2
	O-II	10	272; 112; 106; 92; 86; 78; 75; 72; 38 ; 31	MY2, MY2B, MY3B, <i>M. odoratus</i>	4
22 (<i>Chryseobacterium</i> / <i>Elizabethkingia</i>)	O-1	11	61; 57; 53; 48; 42; 36; 32; 27; 24 ; 23; 21	CH1, CH4, CH4B	3
	O-2	17	90; 87; 83; 67; 62; 55; 50; 45; 40; 36; 34; 33; 30 ; 28 ; 26 ; 24 ; 21	CH1B	1
	O-3	11	135; 115; 105; 91; 57; 53; 47 ; 36; 33; 31; 21	CH2, CH2B	2
	O-4	17	95; 84; 69; 59; 56; 47; 43; 40; 37; 33 ; 31; 28; 27; 24 ; 22 ; 21; 20	CH3, CH10	2
	O-5	11	42; 38; 36; 32; 29; 27; 26 ; 25; 23 ; 22; 18	CH5, CH6, CH7, CH8, CH9	5
	O-6	11	108; 83; 62; 60; 56; 49; 47; 41 ; 31 ; 28; 21	CH11, CH12	2
	O-7	10	200; 147; 120; 86; 83; 74; 46 ; 30; 22 ; 21	CH13	1
	O-8	2	45 ; 22	CH14	1
	O-9	6	61; 45; 37; 36; 24; 21	CH15, CH16, CH17, CH18; CH19	5
	O-10	4	66; 49; 26 ; 22	CH21	1
	O-11	17	168; 146; 119; 105; 88; 80; 76; 62; 55; 47; 44; 34; 32; 30; 29; 28 ; 26	CH23, CH24	2
	O-12	4	42; 32 ; 30 ; 29	CH25, CH26, CH28	3
	O-13	7	111; 103; 91; 86; 54 ; 43 ; 34	CH27	1
	O-14	11	111; 93; 88; 65; 59 ; 48 ; 32; 29; 24; 23; 21	CH22, CH29	2
	O-15	5	60; 57; 44; 32; 21	CH30	1
	O-16	10	108; 91; 85; 59; 48 ; 32; 28; 27; 23; 21	CH33	1
	O-17	8	56; 54 ; 51 ; 48; 40; 35; 23; 21	CH34	1
	O-18	1	31	<i>C. balustinum</i>	1
	O-19	6	53 ; 40; 35; 31; 29; 26	<i>C. gleum</i>	1
	O-20	14	110; 96; 59; 54 ; 51 ; 45 ; 39 ; 36; 33; 31; 30; 29; 27; 25	<i>C. indologenes</i>	1
	O-21	4	53; 49; 45; 31	<i>E. meningoseptica</i>	1
	O-22	19	108; 96; 89; 80; 72; 62; 59; 49; 43; 40; 38; 34 ; 31; 29; 28; 27; 26; 24; 20	<i>C. indoltheticum</i> <i>C. joostei</i>	2

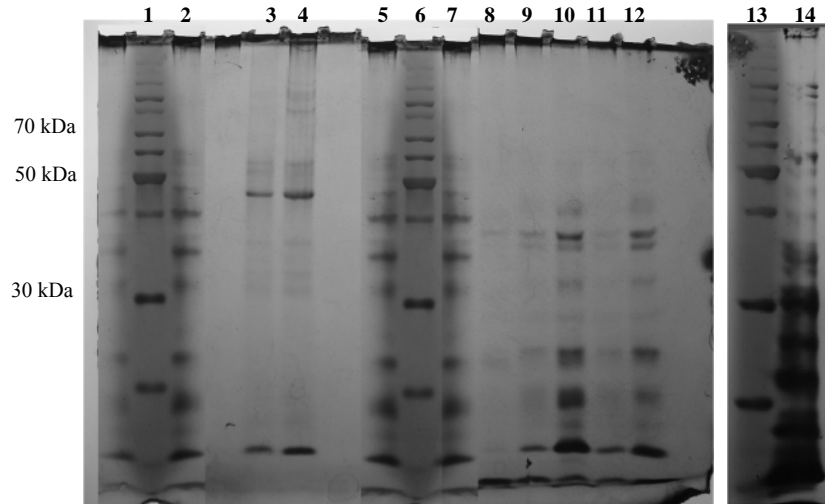


Figure 2. 47 Electrogram displaying outer membrane protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lanes 1, 6 and 13: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada); lanes 2-5: CH1, CH2, CH2B, CH4; lanes 7-12: CH4B, CH5, CH6, CH7, CH8, and CH9, respectively; and lane 14: CH1B.

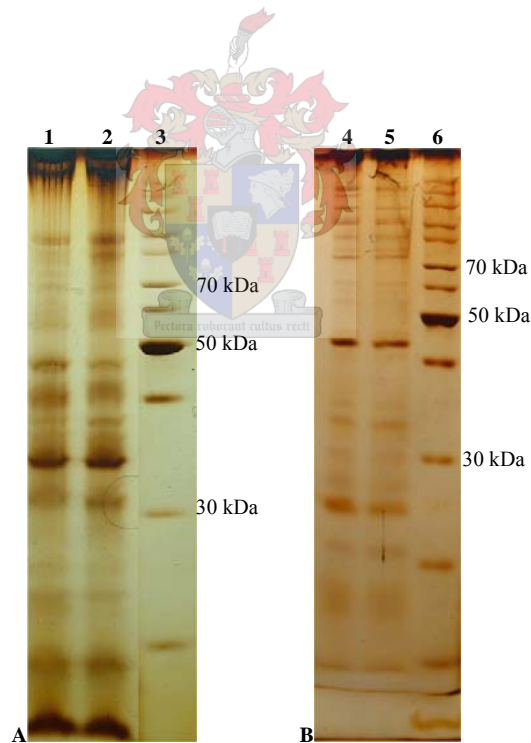


Figure 2. 48 Electrogram displaying outer membrane protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lanes 1 and 2: CH3 and CH10; lanes 3 (A) and 6 (B): PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada); and lanes 4 and 5: CH23 and CH24, respectively.

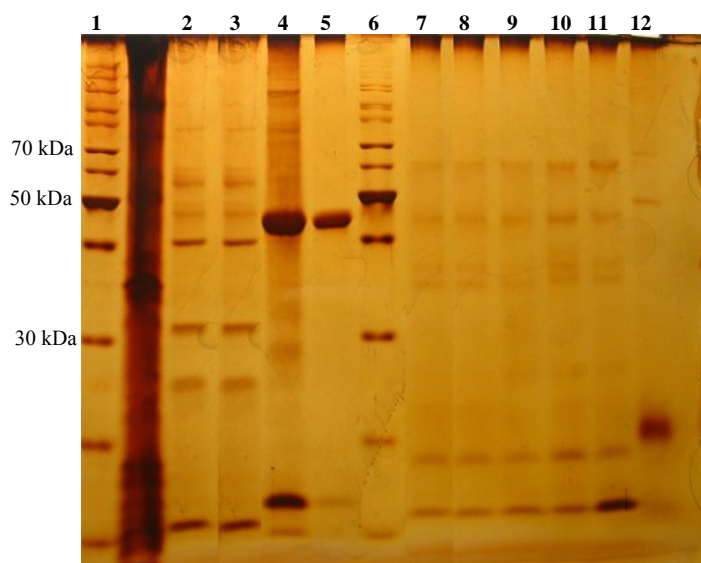


Figure 2. 49 Electrogram displaying outer membrane protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lanes 1 and 6: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada); lanes 2-5: CH11, CH12, CH13, CH14; and lanes 7-12: CH15, CH16, CH17, CH18, CH19, and CH21, respectively.

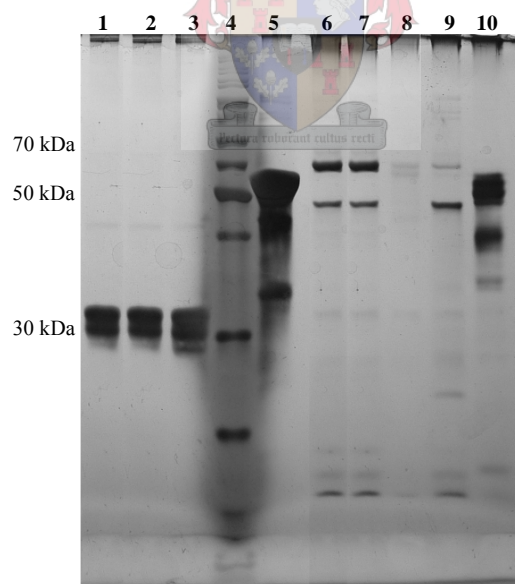


Figure 2. 50 Electrogram displaying outer membrane protein profiles of *Chryseobacterium* and *Elizabethkingia* spp. study isolates. Lanes 1-3: CH28, CH25, CH26; lane 4: CH PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/20/15/10 kDa) (Fermentas, Canada); and lanes 5-10: CH27, CH28, CH22, CH29, CH30, CH33, and CH34, respectively.

Clusters obtained by the Pearson product-moment correlation coefficient are shown in dendograms (Figs. 2.53-2.54). For the *Myroides* and *Empedobacter* spp. isolates a total of 5 clusters were established at a maximum similarity level of 91.5% (Fig. 2.53). The collection of isolates was divided into 2 major clusters (A and B) at ~70% similarity. Cluster A contained two distinct branches with the first branch containing study isolates MY1, MY2 and MY3, and the second containing study isolates MY2B and MY3B, and cluster B contained reference strain *M. odoratus* NTCC 11036. Isolates grouped together in group O-I by visual analysis, were separated by the two major clusters. Isolates MY1 and MY3 displaying identical profiles in visual analysis displayed 82.5% similarity in GelCompar analysis (Fig. 2.53). These differences may be ascribed to differences in protein band intensities and gel alignment, which leads to different clustering by GelCompar analysis compared to visual clustering.

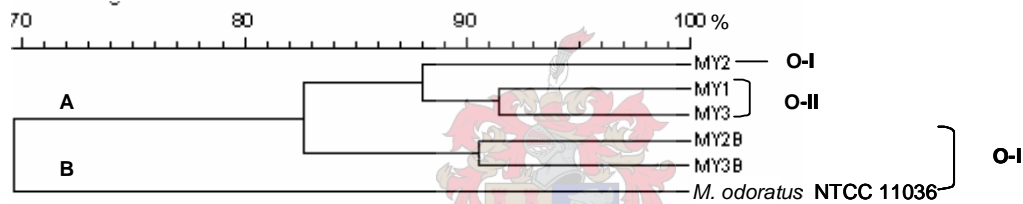


Figure 2. 51 Dendrogram generated by cluster analysis of outer membrane protein profiles of the *Myroides* and *Empedobacter* spp. isolates and reference strain.

For the *Chryseobacterium* and *Elizabethkingia* spp. isolates a total of 38 clusters were established at a maximum similarity level of 100% (Fig. 2.54). The collection of isolates was divided into 7 major clusters (A to G) at 16% similarity. Groups O-1, O-3, O-5, O-6, O-11, O-12 and O-14, respectively, contained isolates displaying identical protein profiles during visual analysis, and displayed similarity $\geq 81\%$ between isolates in the respective groups (Table 2.8; Fig. 2.54). Isolates grouped together in groups O-4 and O-9, were separated by dendograms analysis, but remained within clusters D and A, respectively. Variation of clustering by dendograms analysis and visual analysis may be attributed to differences in protein band intensities and gel alignment. Unlike RAPD and WCP analysis, the reference strains did not cluster together but were dispersed more widely amongst the study isolates. In accordance to the high discriminatory index

calculated for OMP analysis (Table 2.10), dendrograms analysis indicated that the isolates displayed far more genetic heterogeneity by OMP subtyping than by RAPD analysis and WCP analysis.

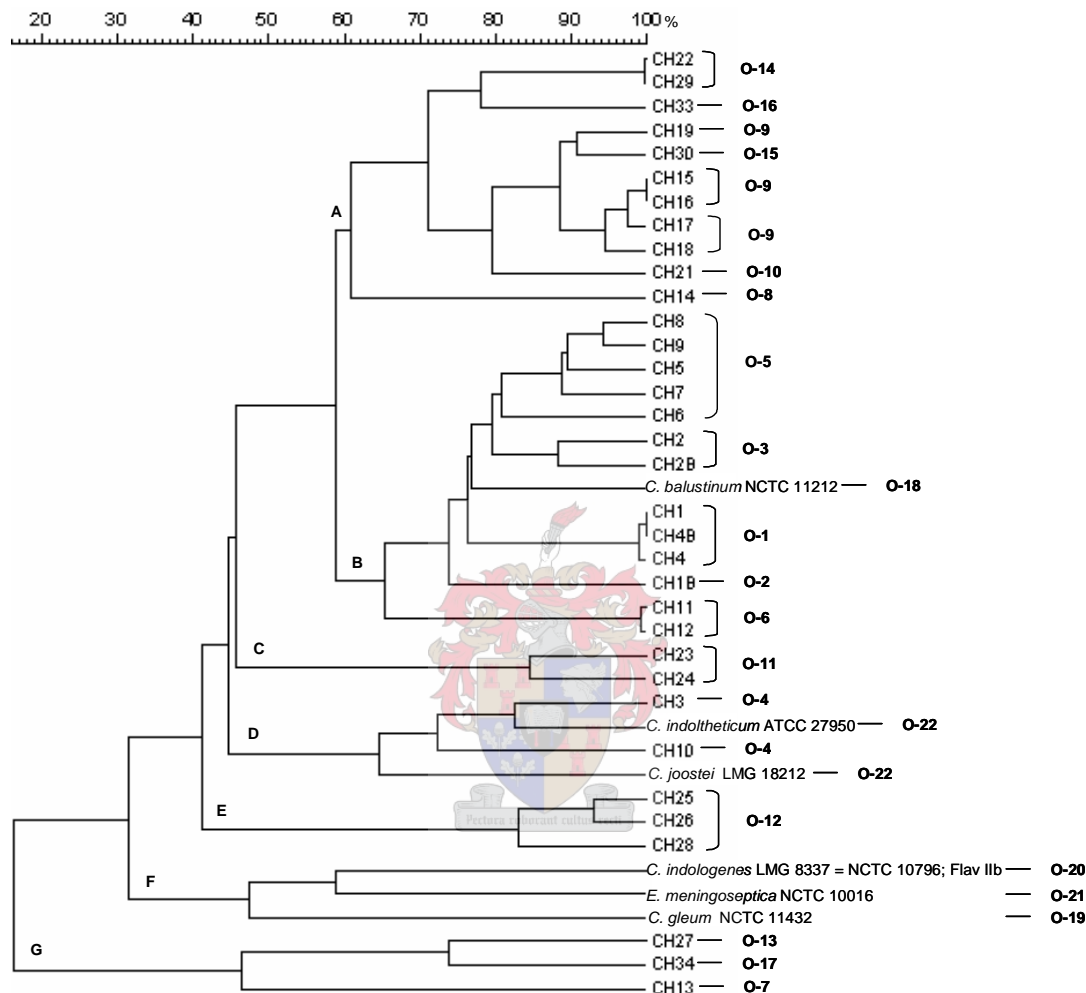
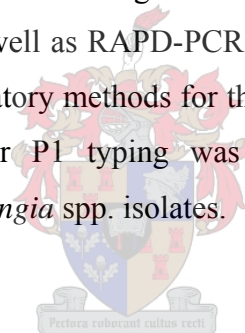


Figure 2. 52 Dendrogram generated by cluster analysis of outer membrane protein profiles of the *Chryseobacterium* and *Elizabethkingia* spp. isolates and reference strains.

2.3.8 Typeability, reproducibility and discriminatory ability of typing methods

Since all the *Myroides* and *Empedobacter* spp. isolates were typeable by PCR-RFLP analysis, RAPD primer P2 fingerprinting, WCP analysis and OMP analysis (Table

2.9), the typeability index was determined at 100% for each respective typing method. Not all of these isolates were typeable by RAPD primer P1 fingerprinting and therefore the typeability index was calculated to be 83.3%. All of the *Chryseobacterium* and *Elizabethkingia* spp. isolates were typeable by RAPD fingerprinting, WCP analysis and OMP analysis and therefore the typeability index was determined at 100% for each respective typing method. Since not all of these isolates were typeable by PCR-RFLP analysis, the typeability indices were calculated to be 97.5% and 92.5% for *TaqI* and *MspI*, and *CfoI* restriction analysis, respectively. Using Simpson's index of diversity, the discriminatory indices of 16S rRNA gene PCR-RFLP analysis, RAPD fingerprinting, WCP analysis and OMP analysis, respectively, were calculated and are shown in Table 2.10. OMP analysis appeared to be the most discriminatory method for differentiating between *Myroides* and *Empedobacter* spp. isolates and *Chryseobacterium* and *Elizabethkingia* spp. isolates. 16S rRNA gene PCR-RFLP analysis using restriction endonucleases *CfoI* and *MspI* as well as RAPD-PCR with primer P1, and WCP analysis appeared to be the least discriminatory methods for the *Myroides* and *Empedobacter* spp. isolates, and RAPD-PCR primer P1 typing was the least discriminatory for the *Chryseobacterium* and *Elizabethkingia* spp. isolates.



2.4 Discussion

The increased isolation of members of the genus *Chryseobacterium* and *Elizabethkingia* by fish pathology laboratories (Bernardet *et al.*, 2005) has highlighted the need to investigate the yellow-pigmented *Flavobacteriaceae* from aquatic sources. Yellow-pigmented isolates belonging to the genera *Chryseobacterium*, *Elizabethkingia*, *Myroides* and *Empedobacter* were isolated from a diversity of aquaculture fish during the period 2003 – 2005. Using physiological and biochemical tests it was not possible to differentiate between the *Myroides* and *Empedobacter* genera, although a closer relatedness to the genus *Myroides* was observed.

Table 2.9 Overall summary of subtypes obtained using diverse molecular typing techniques for differentiation of *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates and reference strains.

Isolates	Biochemical groupings	16S rRNA PCR-RFLP groups			RAPD analysis		PROTEIN CONTENT	
		<i>Cfo</i> I	<i>Msp</i> I	<i>Taq</i> I	Primer I	Primer II	WCP	OMP
<i>Myroides</i> / <i>Empedobacter</i> spp.								
MY1	A	C-I	M-I	T-I	RP1-I	RP2-I	W-I	O-I
MY2	A	C-I	M-I	T-I	RP1-I	RP2-I	W-I	O-II
MY2B	A	C-I	M-I	T-I	RP1-I	RP2-I	W-I	O-II
MY3	A	C-I	M-I	T-I	RP1-I	RP2-I	W-I	O-I
MY3B	A	C-I	M-I	T-I	RP1-I	RP2-I	W-I	O-II
<i>M. odoratus</i> (NCTC 11036)	A	C-I	M-I	T-II	RP1-0*	RP2-II	W-I	O-II
<i>Chryseobacterium</i> / <i>Elizabethkingia</i> spp.								
CH1	2a	C-1	M-1	T-1	RP1-1	RP2-1	W-1	O-1
CH1B	2a	C-2	M-2	T-1	RP1-1	RP2-1	W-1	O-2
CH2	2a	C-2	M-3	T-2	RP1-2	RP2-3	W-2	O-3
CH2B	3b	C-3	M-4	T-5	RP1-2	RP2-3	W-2	O-3
CH3	4a	C-2	M-2	T-11	RP1-1	RP2-2	W-1	O-4
CH4	2a	C-2	M-3	T-2	RP1-1	RP2-2	W-1	O-1
CH4B	4a	C-2	M-3	T-2	RP1-1	RP2-2	W-1	O-1
CH5	2a	C-2	M-2	T-2	RP1-1	RP2-2	W-1	O-5
CH6	2b	C-2	M-5	T-2	RP1-1	RP2-2	W-1	O-5
CH7	2b	C-2	M-6	T-2	RP1-1	RP2-2	W-1	O-5
CH8	2b	C-2	M-3	T-2	RP1-1	RP2-2	W-1	O-5
CH9	2a	C-2	M-7	T-2	RP1-1	RP2-2	W-1	O-5
CH10	5a	C-2	M-3	T-2	RP1-3	RP2-4	W-3	O-4
CH11	3a	C-2	M-12	T-3	RP1-4	RP2-5	W-4	O-6
CH12	3a	C-0*	M-8	T-5	RP1-4	RP2-5	W-4	O-6
CH13	3a	C-2	M-9	T-1	RP1-5	RP2-6	W-5	O-7
CH14	3a	C-0*	M-10	T-4	RP1-5	RP2-6	W-5	O-8
CH15	2b	C-2	M-11	T-1	RP1-6	RP2-7	W-3	O-9
CH16	5b	C-0*	M-0*	T-0*	RP1-6	RP2-7	W-3	O-9
CH17	2b	C-2	M-3	T-1	RP1-6	RP2-7	W-3	O-9
CH18	3b	C-2	M-3	T-1	RP1-6	RP2-7	W-3	O-9
CH19	2b	C-4	M-7	T-1	RP1-6	RP2-7	W-3	O-9
CH21	3b	C-5	M-12	T-3	RP1-7	RP2-8	W-1	O-10
CH22	3b	C-4	M-7	T-1	RP1-8	RP2-9	W-6	O-14
CH23	4c	C-6	M-13	T-6	RP1-9	RP2-10	W-7	O-11
CH24	4b	C-7	M-14	T-6	RP1-9	RP2-10	W-7	O-11
CH25	4b	C-8	M-15	T-7	RP1-10	RP2-11	W-8	O-12
CH26	4b	C-8	M-15	T-7	RP1-10	RP2-11	W-8	O-12
CH27	4c	C-9	M-7	T-1	RP1-10	RP2-12	W-9	O-13
CH28	4b	C-8	M-15	T-7	RP1-10	RP2-11	W-8	O-12
CH29	3b	C-9	M-16	T-1	RP1-11	RP2-13	W-1	O-14
CH30	3b	C-9	M-16	T-1	RP1-11	RP2-13	W-1	O-15
CH33	3b	C-10	M-17	T-8	RP1-11	RP2-13	W-1	O-16
CH34	3b	C-11	M-18	T-9	RP1-12	RP2-14	W-1	O-17
<i>C. balustinum</i> (NCTC 11212)	4c	C-1	M-19	T-10	RP1-13	RP2-15	W-10	O-18
<i>C. gleum</i> (NCTC) 11432	2a	C-13	M-6	T-2	RP1-14	RP2-16	W-11	O-19
<i>C. indologenes</i> (LMG 8337 = NCTC 10796; Flav IIb)	3a	C-1	M-7	T-3	RP1-15	RP2-17	W-12	O-20
<i>C. indoltheticum</i> (ATCC 27950)	2a	C-1	M-7	T-4	RP1-16	RP2-18	W-13	O-22
<i>C. joostei</i> (LMG 18212)	2a	C-13	M-12	T-1	RP1-17	RP2-19	W-14	O-22
<i>E. meningoseptica</i> (NCTC 10016)	3b	C-2	M-20	T-11	RP1-18	RP2-20	W-15	O-21

* Isolates untypeable by respective typing method.

Table 2. 10 Discrimination indices of molecular typing methods used to subtype *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates, respectively.

Typing method	Number of types	Index (%)
<i>Myroides</i> / <i>Empedobacter</i> spp. isolates		
16S PCR-RFLP		
<i>Cfo</i> I	1	0
<i>Msp</i> I	1	0
<i>Taq</i> I	2	33
RAPD PCR		
Primer P1	1	0
Primer P2	2	33
WCP analysis	1	0
OMP analysis	2	53
<i>Chryseobacterium</i> <i>Elizabethkingia</i> spp. isolates		
16S PCR-RFLP		
<i>Cfo</i> I	13	78
<i>Msp</i> I	20	94
<i>Taq</i> I	11	82
RAPD PCR		
Primer P1	18	76
Primer P2	20	93
WCP analysis	15	84
OMP analysis	22	96

Thick capsular material was observed surrounding the *Myroides* spp. cells, and this might potentially facilitate their adherence to surfaces and their ability to interact with other organisms (Brisou, 1959). Although the *M. odoratus* (NTCC 11036) type strain tested negative for growth on 5% NaCl, study isolates were able to grow in the presence of 5% NaCl indicating their ability to survive in marine water and thus infect maricultured fish and cause spoilage of marine fish products, or foods preserved with

high salt concentrations. *Myroides* spp. have been previously isolated from South Atlantic fish species (Engelbrecht *et al.*, 1996) confirming the ability of *Myroides* spp. isolates to proliferate in both freshwater and marine ecosystems. Differences observed between the type strain *M. odoratus* (NTCC 11036) and the present study isolates might be related to differences in the host and ecosystem from which the species originated. The type strain is of clinical origin and the present study isolates would have evolved differently based on the freshwater systems from which they were isolated, where lower temperatures predominate and the nutrient availability continuously fluctuates. It has been suggested that differentiation between the species belonging to this genus through phenotypic, physiological and biochemical tests is not possible (Hugo *et al.*, 2005), and effective discrimination is only possible through carbon source assimilation tests, oxidation assays and differences in DNA G + C content (Vancanneyt *et al.* 1996).

Of the molecular subtyping methods, only OMP profiling allowed the differentiation of the *Myroides* and *Empedobacter* spp. study isolates which clustered together by 16S rRNA-PCR-RFLP typing, RAPD analysis and WCP fingerprinting (Table 2.9). This clustering of isolates or the lack of diversity would explain the low discriminatory indices (Table 2.10) and suggest a genetically homogeneous population. OMP differences might be implicated in the ability of the isolates to adhere to surfaces as this is facilitated by OMP proteins (Ofek *et al.*, 2003).

Based on phenotypic and biochemical test results, isolates displayed characteristics reported for a number of different *Chryseobacterium* and *Elizabethkingia* spp. Study isolates were clustered on the basis of phenotypic and biochemical traits, and compared to previously described species. Physiological observations such as colony morphology and biochemical test results did not correlate which is consistent with previous findings that these organisms are very closely related and molecular characterization is required to distinguish between the isolates (Bernardet *et al.*, 2006). *Chryseobacterium* and *Elizabethkingia* spp. isolates were grouped based on their phenotypic and biochemical test results (Table 2.3). The growth of the isolates clustered into group 2a at 42 °C suggested identification as *C. defluvii* or *C. indologenes*, which are able to grow at this temperature (Bernardet *et al.*, 2006). Isolate CH2B produced a weak yellow pigment and was presumptively identified as *E. meningoseptica*, which displays

this phenotype. The inability to hydrolyse starch suggested that isolates in groups 3 and 5b were potentially *C. balustinum*, *C. indoltheticum*, *C. scophthalmum*, *C. vrystaatense* or *C. piscium* as these species were previously described as being devoid of the ability to hydrolyse starch (de Beer *et al.*, 2006; de Beer *et al.*, 2005; Kim *et al.*, 2005a; Campbell and Williams, 1951; and Harrison, 1929). Isolates in group 3a and 5b could not grow above temperatures of 37 °C, but this phenotype could not be used to further distinguish the groups as it was shown that growth at this temperature varies significantly among *Chryseobacterium* species (Bernardet *et al.*, 2006). The growth of group 4 isolates in 5% NaCl, suggested *C. piscium* or *C. taeanense* (de Beer *et al.*, 2006). Since the isolates in subgroup 4b were unable to hydrolyse starch, which suggested similarity to *C. piscium* (de Beer *et al.*, 2006).

Contrary to expectations, it was not possible to identify *Chryseobacterium* and *Elizabethkingia* spp. isolates based on similarity of RAPD, WCP and/or OMP profiles of reference strains and study isolates. It was, however, possible to cluster isolates with reference strains by 16S rRNA-PCR-RFLP analysis. However, using the different subtyping methods allowed the clustering as well as differentiation of study isolates (Table 2.9). OMP analysis and 16S rRNA PCR RFLP analysis using *MspI* allowed the differentiation of isolates clustered together by *CfoI*, *TaqI*, RAPD analysis and WCP profiling (Table 2.9). Discriminatory indices ranged from 76 – 96% when *Chryseobacterium* and *Elizabethkingia* isolates were characterized, suggesting the ability of OMP analysis, RAPD PCR with primer P2 and 16S rRNA-PCR-RFLP analysis with *MspI* to discriminate between isolated species belonging to these genera (Table 2.10). Although it was not possible to correlate RAPD profiles with that of specific reference strains, differentiation between study isolates, as well as reference strains was possible. The presence of common bands in profiles obtained with RAPD primers P1 and P2 could potentially be used to identify genes or gene sequences which will allow rapid molecular-based identification of *Chryseobacterium* and *Elizabethkingia* species (diagnosis) or their species group (species assignment).

Chiu *et al.* (2000) used the RAPD technique to differentiate *E. meningoseptica* from bacteria of other genera used in their study. They observed that RAPD profiling allowed the differentiation of *E. meningoseptica* from different geographic areas (Chiu *et*

al., 2000). *Chryseobacterium* spp. isolates from salmon (isolates CH29 and CH30) were identical to a trout isolate (CH33) with regards to their biochemical, RAPD and WCP subtypes. Isolates from different fish species appeared to be genetically distinct although clusters were observed with the fish species grouping. *Chryseobacterium* isolates CH16-CH19 isolated from fish from Malawi shared RAPD, WCP and OMP profiles with CH15, which was isolated from trout eggs from Stellenbosch. RAPD primers used in the present study were identical to those used in the polyphasic study of *Chryseobacterium* strains isolated from diseased fish species by Bernardet *et al.* (2005). According to Bernardet *et al.* (2005), delineation of species was possible through RAPD analysis, although a very high overall similarity of *Chryseobacterium* and *Elizabethkingia* strains was demonstrated. This increases the difficulty in delineating new species to these genera.

Phenotypic typing techniques did not allow for grouping of the study isolates with any of the reference strains and the study isolates could therefore not be characterized to species level through any of the molecular techniques. WCP analysis generated 15 protein profiles, and although not as discriminatory as RAPD analysis, similar groupings were observed. Similar results were obtained by Bernardet *et al.* (2005), who demonstrated delineation of clusters of species belonging to the genus *Chryseobacterium* through RAPD PCR analysis and WCP analysis. OMP analysis generated 21 protein profiles, and allowed for the differentiation between isolates clustered together in groups C-2, T-2, RP1-1, RP2-2 and W-1 similar to *CfoI* restriction analysis (Table 2.9). OMP analysis also allowed for the discrimination of study isolates CH29 and CH30, which could not be accomplished using of the other typing techniques (Table 2.9).

Although in most cases, visual groupings in profiles corresponded to clusters obtained following GelCompar analysis, it is possible to explain the discrepancy between the two methods. In visual analysis band intensity was disregarded in favour of presence/absence of bands which allowed for a more accurate assessment of clusters sharing similar or identical RAPD profiles. However, GelCompar software takes variability in band intensities into account when delineating clusters, which may account for differences in groups obtained by visual grouping and clusters obtained from GelCompar analysis. In addition, the presence of primary, secondary and tertiary amplicons (Bassam *et al.*, 1992) and poor gel alignment due to slight differences in

electrophoretic conditions could have affected the clustering of RAPD profiles. Differences in band intensities resulting from both variation in levels of protein expression and the concentration effect and slight differences in SDS-PAGE leading to poor gel alignment, may explain the differences in groups obtained by GelCompar analysis compared to clusters established during visual analysis of profiles observed for WCP analysis and OMP analysis. For the *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates, reference strains were clustered into separate clusters than the through GelCompar analysis of RAPD profiles and WCP profiles. However, in accordance to the high discriminatory index obtained for OMP analysis (Table 2.10), differentiation was made between the reference strains which were clustered amongst the study isolates during GelCompar analysis.

The most effective antibiotics for *Myroides* and *Empedobacter* spp. isolates were azithromycin, cefoxitin, cotrimoxazole, naladixic acid, norfloxacin and sulphamethoxazole due to 100% susceptibility demonstrated by the study isolates (Table 2.6; Fig. 2.37). All of the isolates had very high MAR indices, indicating that these isolates have previously been exposed to antibiotics, although the isolates from the Blue tilapia aquaculture system had not been treated with antibiotics (Table 2.1). This suggests that the isolates acquired resistance genes from another source, or that they possess a high intrinsic antibiotic resistance. Either scenario poses a great threat in veterinary or human medicine, as exposure to antibiotics could lead to a multiple drug resistance (MDR) phenotype which would make treatment of any infection nearly impossible. Varying levels of susceptibility and resistance was observed for the *Chryseobacterium* and *Elizabethkingia* spp. isolates. The only antibiotic to which the isolates displayed 100% susceptibility was piperacillin-tazobactam (Table 2.6; Fig. 2.38). Piperacillin/tazobactam was also among the most active agents against the genera *Chryseobacterium* and *Elizabethkingia* in a study by Kirby *et al.* (2004). High MAR indices were recorded for seven isolates isolated from Blue tilapia aquaculture systems, and one isolate from a rainbow trout system. Unlike the trout systems, tilapia aquaculture systems have not previously been exposed to antibiotics, and therefore these isolates are expected to have contaminated the aquaculture system from other environments where antibiotics had been used previously or isolates have acquired resistance genes while in

aquaculture system. No specific correlation could be made between antimicrobial susceptibility profiles displayed by the study isolates and subtypes generated by PCR RFLP, RAPD, WCP or OMP typing.

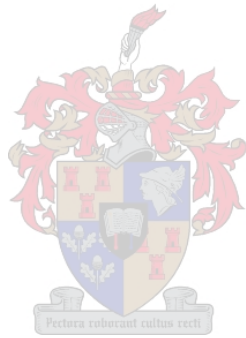
To evade the actions of β -lactam antibiotics, bacteria produce hydrolytic enzymes referred to as β -lactamases (Zeba *et al.*, 2005). Hydrolyzing enzymes are commonly found in the genera *Myroides*, *Empedobacter*, *Chryseobacterium* and *Elizabethkingia* (Michel *et al.*, 2005). β -lactamase enzymes that have the ability to hydrolyse carbapenem antibiotics are called metallo- β -lactamases (M β L) and have been reported in the genera *Chryseobacterium*, *Elizabethkingia* and *Myroides* (Zeba *et al.*, 2005; Woodford *et al.*, 2000). Chromosomal-encoded M β Ls are inherent to few bacterial species, including the flavobacteria (Bush, 1998; and Livermore, 1997). M β Ls have been reported for *C. indologenes* (Zeba *et al.*, 2005; and Bellais *et al.*, 1999), *E. meningoseptica* (Rossolini *et al.*, 1998) and *M. odoratus* (Sato *et al.*, 1985). The M β L enzymes identified by Zeba *et al.* (2005) were able to hydrolyse common β -lactam antibiotics such as ampicillin, amoxicillin and cefuroxime, but was unable to act on ceftazidime. *Chryseobacterium* and *Elizabethkingia* spp. isolates CH1-8 and CH34 resistant to imipenem in the present study displayed similar susceptibility/resistance profiles results as that reported by Zeba *et al.* (2005). Therefore, it is not unlikely that these isolates possess M β Ls. It has been proposed that species belonging to the genera *Chryseobacterium*, *Elizabethkingia* and *Myroides* may represent a reservoir of diverse M β Ls, which could potentially spread to Gram-negative bacteria of greater clinical significance (Woodford *et al.*, 2000). Carbapenems are increasingly used for the treatment of infections caused by MDR Gram-negative organisms (Bush, 1998), and thus the presence of M β L producing organisms in the environment could pose a threat to treatment of human infection.

If an organism is able to hydrolyse β -lactam antibiotics from various groups, it is said to possess extended spectrum β -lactamases (ES β L), and these have been found to occur in *Chryseobacterium* and *Elizabethkingia* spp. (Bellais *et al.*, 2002). Bellais *et al.* (2000) identified chromosomally-encoded ES β Ls in *E. meningoseptica*, and ES β Ls have also been described for *C. gleum* (Bellais *et al.*, 2002). In the present study, isolates identified as possibly carrying ES β Ls genes included: CH1-8 (resistant to all penicillins, cefuroxime and imipenem), CH34 (resistant to oxacillin, cefuroxime and imipenem), and

MY2B (resistant to all penicillins, ceftriaxone, cefuroxime, and piperacillin/tazobactam) (Table 2.6).

Chryseobacterium, *Elizabethkingia*, *Myroides*, and *Empedobacter* spp. isolates are occasionally involved in human clinical outbreaks (Lee *et al.*, 2006; Bernardet *et al.*, 2006; Hugo *et al.*, 2005; Hoque *et al.*, 2001; Yagci *et al.*, 2000; Hseuh *et al.*, 1997; Hseuh *et al.*, 1996; and Holmes *et al.*, 1979). According to Bernardet *et al.* (2005), the acquisition of resistant strains from an aquacultural environment by immunocompromised humans should not be underestimated. Although their virulence for fish has not been proven, the present study confirms the report by Bernardet *et al.* (2005) that *Chryseobacterium* spp. strains are widely distributed in aquacultural settings and may be easily isolated from diseased fish. The presence of different strains belonging to different clusters occurring in the same fish farm or sampling site was also observed for study isolates. *Chryseobacterium* spp. most likely are harboured as part of fish flora but act as opportunistic pathogens when fish undergo severe stress. In addition to being present on fish, Leonard *et al.* (2000) reported the ability of heterotrophic bacteria to exist in biofilms in aquaculture recirculation systems. These biofilm-associated bacteria thus serve as a potential reservoir or source for future infection or re-infection.

Given their potential pathogenic role in aquaculture systems, the ability of study isolates to adhere to surfaces and form biofilms was investigated. This involved investigating the capacity to form biofilms and an exploration of surface characteristics and their role in the biofilm-forming phenotype.



CHAPTER THREE

Adherence and cell-surface characterization of *Chryseobacterium*, *Elizabethkingia*, *Myroides* and *Empedobacter* spp. isolates obtained from aquaculture systems

3.1 Introduction

Microorganisms display a strong tendency to colonize surfaces. With the subsequent multiplication, adherence of additional bacterial species and production of EPS, a complex microbial biofilm community is formed on the colonized surface. Aquatic microorganisms are well known for their ability to form biofilms (Rickard *et al.*, 2003b; Webb *et al.*, 2003; Donlan, 2002; Bell, 2001; and Stickler, 1999).

Adhesion is a prerequisite for the formation of biofilms on surfaces (Coquet *et al.*, 2002a) and may be influenced by various substrate, cell-surface and environmental factors (Table 3.1). Formation of a biofilm is preceded by the attachment of primary colonizers, mediated through physico-chemical interactions with components of an adsorbed, organic conditioning film present on the substratum (Rickard *et al.*, 2003b). In addition to physico-chemical interactions, initial adherence of microorganisms to surfaces may also involve protein and polysaccharide factors, and is thus a very complex process (Cerca *et al.*, 2005). Physico-chemical interactions include various long-range non-specific interactions such as Lifshitz-van der Waals forces, electrostatic forces, acid-base interactions, and Brownian motion forces (Azeredo and Oliveira, 2000; and van Oss and Giese, 1995). Depending on the sum of the different non-specific interactions, microorganisms will be attracted or repelled upon reaching a surface (Fonseca *et al.*, 2001). Initial attachment of cells is facilitated by expression of cell-surface polymers, which alter cell surface properties (Rickard *et al.*, 2004a). Various mechanisms of attachment have been described and these mechanisms utilize different cell components including cell-surface structures such as flagella, pili, fimbriae, OMPs and cell wall polysaccharides such as capsules (Donlan, 2002; Davey and O'Toole, 2000; and Stickler, 1999). Sugars such as glucose, glucuronic acids, galactose, and fructose that are commonly found in the composition of capsular structures have been proposed to play a

capital role in the mechanisms of adherence (Brisou, 1959). In addition, many of these structures are important components of bacterial motility and non-motile mutants of *P. aeruginosa* have been shown to lack biofilm-forming ability, compared to wild type cells (Stickler, 1999).

Table 3.1 Summary of the factors influencing adherence and biofilm formation (Wimpenny *et al.*, 2000).

Influential factors	Descriptions
Genotypic factors	The specific genotype of the organism. Expression of genes encoding surface properties. Expression of signalling molecules. Formation of EPS. Organism growth dynamics; specific growth rate, lag periods, affinity for substrates. Expression of genetic factors not directly connected to biofilm formation (e.g. motility).
Physico-chemical factors	Phase interface (combinations of solid, liquid and gaseous). Substratum composition and roughness. Substrate composition, concentration, gradient. Temperature, pH, water potential, pressure, oxygen supply and demand, radiation effects.
Stochastic processes	Initial colonisation: attachment, detachment. Random changes in abiotic and biotic factors.
Deterministic phenomena	Specific interactions between organisms: competition, neutralism, cooperation and predation.
Mechanical processes	Shear due to laminar or turbulent flow conditions; abrasion; logistic restrictions. Addition or removal of biotic or abiotic components to a biofilm system; e.g. import of sand, clay minerals or organic detritus into a biofilm structure. Sloughing off of biomass, release of individual cells.
Import-export	
Temporal changes	Diurnal or annual periodic changes in biotic and abiotic environment; e.g. light, temperature pH, PO_2 . Irregular changes due to unforeseen events

Cell-surface hydrophobicity is considered important in adhesion because hydrophobic interactions tend to increase with increasing non-polar nature of one or both surfaces involved (i.e., microbial cell surface and the substratum surface). Most bacteria are negatively charged but still contain hydrophobic surface components (Donlan, 2002). Microorganisms have been found to attach more rapidly to hydrophobic, non-polar surfaces such as plastics than to hydrophilic materials such as glass or metals (Bendinger *et al.*, 1993; Pringle and Fletcher, 1983; and Fletcher and Loeb, 1979). Costerton *et al.* (1995) suggested that the overall hydrophobicity of a bacterium could serve as a good predictor of the surface that an organism might colonize. There is a difference of opinion

concerning the importance of cell-surface hydrophobicity in adherence of bacterial cells to surfaces. Some authors have shown that hydrophobic interactions contribute in the adherence process, Zita and Hermansson (1997) found a significant correlation between the hydrophobicity of *E. coli* cells and their adherence ability to activated sludge flocs, and Pasmore *et al.* (2001) demonstrated that increased hydrophobicity of *P. aeruginosa* cells results in increased biofilm initiation to surfaces in water-processing environments. Others have found no correlation between hydrophobicity and adherence (Cerca *et al.*, 2005; Chae *et al.*, 2006; and Balebona *et al.*, 2001). Chae *et al.* (2006) found that there was no correlation between the hydrophobicity and attachment of *L. monocytogenes* to glass surfaces, while Cerca *et al.* (2005) found that hydrophobicity had no influence on the adherence of *Staphylococcus epidermidis* to surfaces in the clinical environment. In addition, the adhesion of pathogenic *Vibrio* spp. to fish mucus or cells was found not to be mediated by cell surface hydrophobicity (Balebona *et al.*, 2001).

Several methods have been used to determine hydrophobicity of bacterial cells such as bacterial adherence to hydrocarbons (BATH) (Rosenberg *et al.*, 1980), hydrophobic interaction chromatography (HIC) (Donlon and Colleran, 1993), aggregation in the presence of different salt concentrations (Lindhal *et al.*, 1981), adherence to nitrocellulose filters (NCF) (Lachica and Zink, 1984) and contact angle (van Loosdrecht *et al.*, 1987). The salting aggregation test (SAT) provides a measure of overall surface hydrophobicity, while the bacterial adherence to hydrocarbon (BATH) assay indicates the presence of hydrophobic domains on an otherwise hydrophilic cell surface (Sorongon *et al.*, 1991; and van der Mei *et al.*, 1987). With the BATH assay, a cell suspension is mixed with a hydrocarbon for a predetermined time period to allow optimal interaction of the bacteria with the hydrocarbon phase. As a result, cells may remain in the liquid phase or partition either into the liquid-hydrocarbon interface or into the hydrocarbon phase, depending on their hydrophobicity (Pembrey *et al.*, 1999). SAT assay is based on the more hydrophobic bacterial cells associating with lower concentrations of ammonium sulfate and precipitating. Hydrophobicity is determined through visual analysis, and documented as the concentration of ammonium salt at which the bacterial cell precipitates (Rozgonyi *et al.*, 1985).

Fluid flow velocity (and associated shear rates) has been suggested to be important in the development of biofilm community structure (Beyenal and Lewandowski, 2002) and govern the abilities of individual species to immigrate to biofilms and to colonize new surfaces (Cloete *et al.*, 2003; and Stoodley *et al.*, 1999). Various observations have indicated that high fluid velocities with associated high shear forces lead to the development of less diverse biofilm communities as compared to those developed at lower shear rates (Rickard *et al.*, 2004b; Cloete *et al.*, 2003; Rickard *et al.*, 2003b; Liu and Tay, 2002; and Soini *et al.*, 2002). An increase in surface roughness has been observed to increase the extent of microbial colonization (Characklis *et al.*, 1990). This is because shear forces are diminished, and surface area is higher on rougher surfaces (Donlan, 2002).

The composition of the substratum and composition and concentration of substrates has also been implicated in biofilm-forming abilities of microorganisms such as *L. monocytogenes*, which displays increased attachment in minimal media (Kim and Frank, 1994; and Harvey *et al.*, 2006) and *E. coli* O517:H7 which has been reported to form a biofilm only in low-nutrient media (Dewanti and Wong, 1995). However, other bacterial strains, *E. coli* K-12 and *V. cholerae*, will not form biofilms in minimal media (Watnick *et al.*, 1999; and Pratt and Kolter, 1998). In addition, factors other than adherence such as cell-size have been directly correlated to substrate concentration for anaerobic biofilm-forming bacteria (Szewzyk and Schink, 1987).

Efforts are continually being made to improve assays for investigating all aspects of adhesion and adherence. The microtitre-plate adherence assay is a rapid method for investigating biofilm-forming abilities of microorganisms and involves the investigation of bacterial attachment to the surface of multiwell plates (O'Toole *et al.*, 1999). Using this technique, large quantities of bacterial strains can be investigated for their biofilm formation capability (O'Toole *et al.*, 1999). PVC or polystyrene microtitre plates are commonly used, although polypropylene, polycarbonate plastic or borosilicate glass have also proven effective substrates for microtitre adherence assays (O'Toole *et al.*, 1999). Although various modifications of this technique have been described (Gavin *et al.*, 2003; Croxatto *et al.*, 2002; and O'Toole *et al.*, 1999), the modified microtitre plate assay

described by Stepanovic *et al.* (2000) has been found to be a more accurate and objective assay (Flemming *et al.*, 2006).

A survey of the literature revealed the presence of *Chryseobacterium/Elizabethkingia* and/or *Myroides* and *Empedobacter* spp. isolates in biofilms from different sources (Pavlov *et al.*, 2004; McBain *et al.*, 2003; Bremer *et al.*, 2001; Hoque *et al.*, 2001; and Tide *et al.*, 1999). However, not much is known about the factors involved in initiating biofilm-formation by these isolates. Thus, all study isolates were assayed for their ability to adhere to polystyrene using the microtitre plate assay. A clear picture of attachment cannot be obtained without considering the effects of substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium, and cell-surface properties. In the present study, the effect of some of these factors including hydrodynamics and characteristics of the aqueous medium, and effects of incubation temperature, on the initial adherence ability of the study isolates to PVC microtitre plates as substratum, were investigated.

3.2 Materials and Methods

3.2.1 Microtitre plate adherence assays

Microtitre plate adherence assays (Stepanovic *et al.*, 2000) were conducted to determine the adherence abilities of the study isolates. *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. study isolates were cultured O/N in EAOB, pelleted by centrifugation (12 000 rpm, 2 min) and washed three times in PBS (pH 7.2). Washed cells were resuspended in PBS and standardized to a turbidity equivalent to a 0.5 McFarland standard. Wells of sterile 96-well U-bottomed microtitre plates (Deltalabs, S.L, Barcelona, Spain), were filled with 90 µl of growth media, EAOB or Tryptic soy broth (TSB), and inoculated with 10 µl of the standardized cell suspension, in triplicate. Two negative control wells, containing only broth and PBS were included in each run, and a *Vibrio* spp. isolate was used as a positive control. Plates were placed on a horizontal shaker and/or benchtop to simulate dynamic and static conditions, respectively, and incubated aerobically at room temperature ($21\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) and/or $37\text{ }^{\circ}\text{C}$. Following incubation, contents of each well were aspirated, and washed three times with

150 μ l of sterile PBS. Plates were vigorously shaken in order to remove all non-adherent bacteria. Remaining attached bacteria were fixed for 15 min with 150 μ l of 99% methanol, and left to dry. Plates were stained for 5 min with 150 μ l crystal violet. Excess stain was rinsed off by placing the plate under running tap water. After the plates had been air dried, the dye bound to the adherent cells were resolubilized with 150 μ l 33% (v/v) glacial acetic acid. Optical density (OD) of each well was taken at 595 nm using an automated microtitre-plate reader (Microplate Reader model 680, BioRad Laboratories Inc., Hercules, California). Tests were done in triplicate on three separate occasions and the results averaged (Stepanovic *et al.*, 2000). Isolates were classified into the following adherence categories non-adherent, weakly adherent, moderately adherent, or strongly adherent, according to criteria described by Stepanovic *et al.* (2000). The cut-off OD (OD_c) for the microtitre plate test was defined as three standard deviations above the mean OD of the negative control.

Isolates were classified as follows:

$OD \leq OD_c$	non-adherent,
$OD_c < OD \leq 2 \times OD_c$	weakly adherent,
$2 \times OD_c < OD \leq 4 \times OD_c$	moderately adherent,
$4 \times OD_c < OD$	strongly adherent.

A second method of assessing the biofilm-forming capacity for each of the study isolates was expressed relative to the average value of all isolates as follows:

$$\text{Relative biofilm capacity} = [A_x - A_0] / \left[\sum_{n=1}^y (A_n - A_0) / y \right],$$

where, A_x = absorbance at 595 nm for isolate x , A_0 = absorbance for uninoculated growth medium, and y = number of isolates in sample (Van Houdt *et al.*, 2004).

Adherence was assayed by varying parameters including: incubation temperature [room temperature ($21 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$) vs $37 \text{ }^\circ\text{C}$], incubation time (16 h, 24 h, 36 h and 42 h), growth media [EAOB (nutrient-poor media) vs TSB (nutrient-rich media)] and static vs dynamic incubation, in order to determine optimal conditions for *in vitro* biofilm formation.

3.2.1.2 *Induction of adherence*

The standard microtitre adherence test (Stepanovic *et al.*, 2000) was modified to determine the ability of various aquaculture, food and/or human pathogens to induce adherence by selected study isolates. One *Myroides* and *Empedobacter* spp. isolate (MY1), and seven *Chryseobacterium* and *Elizabethkingia* spp. isolates (CH2B, CH8, CH15, CH23, CH25, CH26, CH34) were selected for the induction experiments based on their adherence in EAOB and TSB under static conditions at room temperature (as described in section 3.2.1). The panel of microorganisms whose induction abilities were assayed included *Aeromonas hydrophila*, *A. salmonicida*, *A. sobria*, *Chryseobacterium* and *Elizabethkingia* spp. isolate CH2B, *E. coli*, *E. tarda*, *L. innocua*, *L. monocytogenes*, *P. aeruginosa*, *S. enterica* serovar Arizonae, *F. johnsoniae*-like spp. isolate YO59, and a *Vibrio* spp. isolate.

Three-day old cultures of each of the organisms were centrifuged at 2000 rpm for 10 min and filter-sterilised using 0.2 µm filters, in order to obtain cell-free spent medium. Study isolates were prepared as described in section 3.2.1. 10µl of the standardised suspension was added to the microtitre wells containing 100µl TBS and 90µl of the filtered supernatant. Controls included standardised cell suspensions added to TSB and respective filtered supernatant in TSB in order to determine a change in adherence abilities and ensure that the change in adherence was due to induction, respectively. Microtitre plates were incubated on the benchtop at room temperature for three days. Adherence abilities of study isolates were classified into the described four categories (Stepanovic *et al.*, 2000) as well as measuring the relative biofilm-forming capacities (Van Houdt *et al.*, 2004).

3.2.2 *Cell-surface hydrophobicity and capsule presence*

Bacterial surface hydrophobicity of the *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. study isolates was assessed using the bacterial adherence to hydrocarbons (BATH) and modified salting aggregation test (SAT) assays.

3.2.2.1 *Bacterial Adherence to Hydrocarbons (BATH)*

O/N EAOB broth cultures of all study isolates were harvested during the exponential growth phase (18 h old cultures) by centrifugation at 12000 rpm for 2 min. Pellets were washed three times and resuspended in sterile 0.1 M PBS (pH 7). Bacterial suspensions were adjusted to an OD of 0.8 at a wavelength of 550 nm (A_0 of 18^8 CFU/ml), using a DU 640 spectrophotometer (Beckman Coulter, Fullerton, California, USA). Samples (3ml) of bacterial suspensions were placed in glass tubes with 400 μ l of the hydrocarbon, xylene (BDH, VWR International, Leicestershire, UK), equilibrated in a water bath at 25 °C for 10 min, and vortexed (Couquet *et al.*, 2002a; and Rosenberg *et al.*, 1980). After phase separation of 15 min, the optical density (OD_{550}) of the lower aqueous phase was determined (A_1). Values were then expressed as the percentage of cells that adhered to hydrocarbon (A) compared with the control suspension as follows: $A = [(A_0 - A_1)/A_0] \times 100$. Each value represents the mean of experiments done in triplicate and on two separate occasions (Couquet *et al.*, 2002a; and Rosenberg *et al.*, 1980). Study isolates were considered strongly hydrophobic when values were >50%, moderately hydrophobic when values were in the range of 20 – 50%, and hydrophilic when values were <20% (Mattos-Guaraldi *et al.*, 1999). PBS was used as a negative control and a *Vibrio* spp. isolate was used as a positive control.

3.2.2.2 *Salting Aggregation Test (SAT)*

O/N EAOB cultures were harvested, washed twice and resuspended in PBS (pH 7.2) to a final turbidity equivalent to a 0.5 McFarland standard. A series of ammonium sulfate $[(NH_4)_2SO_4]$ solutions in concentrations of 0 M, 0.2 M, 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M and 4 M were prepared (Sorongon *et al.*, 1991), and 350 μ l methyl blue added to each of the 10 ml solutions for better visualization of aggregation (Rozgonyi *et al.*, 1985). Twenty five μ l volumes of the bacterial suspensions were mixed with 25 μ l of the series of $(NH_4)_2SO_4$ solutions on a microscope slide. After 2 min and 4 min of mixing, respectively, on a rocking shaker at ambient temperature, slides were visually examined and scored against a white background. The lowest final concentration of $(NH_4)_2SO_4$ causing aggregation was recorded as the SAT value. Experiments were done in triplicate

on two separate occasions and respective ammonium sulphate concentrations were used as negative controls (Sorongon *et al.*, 1991). Isolates were classified as either hydrophilic or hydrophobic, according to their SAT value. Classification proceeded as follows: < 0.1 M = highly hydrophobic, 0.1 M – 1.0 M = moderate hydrophobicity and > 1.0 M = hydrophilic (Møller *et al.*, 2003).

3.2.3 Statistical analysis

Statistical significance of differences ($p < 0.05$) due to altered variables (temperature, medium and agitation) on the results obtained by the microtitre adherence assays were determined using repeated measures analysis of variance (ANOVA), the means separated by a Bonferroni least significant difference test using Statistica 7 (Statsoft, Tulsa, USA). Relationships between microtitre adherence and hydrophobicity (BATH and SAT assays, respectively), was determined by 2D scatterplot analysis (p -values < 0.05 were considered significant), using Statistica.

3.3 Results

3.3.1 Microtitre adherence assays

The 5 *Myroides* and *Empedobacter* spp. study isolates and 34 *Chryseobacterium* and *Elizabethkingia* spp. isolates were screened for adherence to polystyrene microtitre plate wells following incubation for 24 h at room temperature ($21\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) or $37\text{ }^{\circ}\text{C}$, under static or dynamic conditions in nutrient-rich (TSB) or nutrient-poor (EAOB) media.

At room temperature, biofilm formation ranged from 0.42 (MY2) to 0.65 (MY3B) in EAOB under dynamic conditions, while under static conditions, biofilm formation ranged from 0.40 (MY2) to 0.67 (MY3B) (Table 3.2; Fig. 3.1). At $37\text{ }^{\circ}\text{C}$, biofilm formation ranged from 0.15 (MY3B) to 0.29 (MY3) under dynamic conditions in EAOB, while under static conditions biofilm formation ranged from 0.18 (MY3B) to 0.30 (MY3) (Fig. 3.2). For TSB under dynamic conditions, biofilm formation ranged from 1.22 (MY2) to 1.64 (MY3), while under static conditions, biofilm formation ranged from 1.68

(MY2) to 2.04 (MY1) (Table 3.2; Fig. 3.1). At 37 °C, biofilm formation in TSB under dynamic conditions ranged from 0.20 (MY2) to 0.28 (MY3), while biofilm formation under static conditions ranged from 0.15 (MY3) to 0.26 (MY3) (Fig 3.2). At room temperature, biofilm formation and relative biofilm-forming capacity ≤ 0.1 respectively, were considered a negative result, and none of the isolates were considered to be non-adherent in the presence of TSB, under both static and dynamic conditions (Table 3.3; Figs. 3.3–3.4). Isolates displayed non-adherence or weak adherence and weak to moderate adherence at 37 °C in EAOB and TSB, respectively (Table 3.3; Figs. 3.3–3.4). Significant differences ($p < 0.05$) in biofilm formation were observed when isolates were assayed under static/dynamic conditions at room temperature and 37 °C in nutrient-poor media, respectively (ANOVA, t-tests). All of the isolates had a relative biofilm-forming capacity at room temperature that was significantly ($p < 0.05$) lower than that demonstrated by the *Vibrio* spp. isolate in EAOB but significantly ($p < 0.05$) higher in TSB (Table 3.3; Figs. 3.3-3.4). While the *Vibrio* spp. isolate preferred EAOB to TSB for biofilm formation, all of the *Myroides* and *Empedobacter* spp. isolates displayed stronger adherence in the presence of TSB (Table 3.2-3.3; Figs. 3.1-3.4). The five *Myroides* and *Empedobacter* spp. study isolates displayed overall strong adherence at room temperature with both nutrient-rich and nutrient-poor media, under both static and dynamic conditions (Table 3.3). From these biofilm formation values, greater biofilm formation was documented under static conditions (Table 3.3). By contrast, weak to moderate adherence was observed with nutrient-poor and nutrient-rich media at 37 °C, respectively (Table 3.3).

Table 3. 2 Biofilm formation values and profiles, and relative biofilm-formation capacity of *Myroides* and *Empedobacter* spp. study isolates following incubation at room temperature (~21 °C) under static or dynamic conditions in nutrient-rich (TSB) or nutrient-poor (EAOB) media, respectively.

Isolate	Biofilm formation [‡] (OD ₃₅₅) (Room temperature)								Relative biofilm-forming capacity [#] (Room temperature)							
	EAOB Average (OD±SD)				TSB Average (OD±SD)				EAOB Average (OD±SD)				TSB Average (OD±SD)			
	Dynamic		Static		Dynamic		Static		Dynamic		Static		Dynamic		Static	
<i>Vibrio</i> spp.	1.34 ±0.28	S*	1.61 ±0.18	S	0.11 ±0.00	W	0.14 ±0.07	W	2.03 ±0.46	+	2.40 ±0.24	+	0.02 ±0.00	-	0.03 ±0.00	-
MY1	0.61 ±0.16	S	0.56 ±0.05	S	1.56 ±0.17	S	2.04 ±0.05	S	0.86 ±0.17	+	0.73 ±0.07	+	1.31 ±0.24	+	1.35 ±0.17	+
MY2	0.42 ±0.07	S	0.40 ±0.04	M	1.23 ±0.11	S	1.68 ±0.08	S	0.56 ±0.06	+	0.47 ±0.08	+	1.02 ±0.06	+	1.10 ±0.10	+
MY2B	0.57 ±0.10	S	0.55 ±0.03	S	1.37 ±0.06	S	1.73 ±0.12	S	0.80 ±0.06	+	0.70 ±0.10	+	1.15 ±0.08	+	1.13 ±0.12	+
MY3	0.59 ±0.15	S	0.61 ±0.08	S	1.64 ±0.18	S	1.78 ±0.09	S	0.83 ±0.15	+	0.80 ±0.12	+	1.39 ±0.77	+	1.16 ±0.11	+
MY3B	0.65 ±0.11	S	0.67 ±0.10	S	1.34 ±0.12	S	1.87 ±0.09	S	0.92 ±0.10	+	0.90 ±0.13	+	1.12 ±0.07	+	1.23 ±0.13	+

* Biofilm formation assayed according to Stepanovic *et al.* (2000).

* W/M/S refers to adherence categories displayed by isolates according to criteria described by Stepanovic *et al.* (2000).

Relative biofilm-forming capacity described by Van Houdt *et al.* (2004).

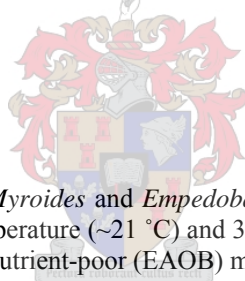


Table 3. 3 Biofilm formation by *Myroides* and *Empedobacter* spp. study isolates (n = 5) following incubation at room temperature (~21 °C) and 37 °C, under static or dynamic conditions in nutrient-rich (TSB) or nutrient-poor (EAOB) media, respectively.

Parameters	Number of isolates									
	Non-adherent		Biofilm formation				Strong		Total	
	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD
21 °C EAOB dynamic	-	-	-	-	-	-	5 (100)	0.57 ±0.12	5 (100)	0.57 ±0.12
21 °C EAOB static	-	-	-	-	1 (80)	0.40 ±0.04	4 (80)	0.60 ±0.07	5 (100)	0.56 ±0.06
21 °C TSB dynamic	-	-	-	-	-	-	5 (100)	1.43 ±0.17	5 (100)	1.43 ±0.17
21 °C TSB static	-	-	-	-	-	-	5 (100)	1.82 ±0.14	5 (100)	1.82 ±0.14
37 °C EAOB dynamic	1 (20)	0.15 ±0.00	4 (80)	0.25 ±0.03	-	-	-	-	4 (80)	0.25 ±0.03
37 °C EAOB static	1 (20)	0.18 ±0.00	4 (80)	0.29 ±0.01	-	-	-	-	4 (80)	0.29 ±0.01
37 °C TSB dynamic	-	-	-	-	5 (100)	0.25 ±0.04	-	-	5 (100)	0.25 ±0.04
37 °C TSB static	-	-	1 (20)	0.15 ±0.00	4 (80)	0.20 ±0.04	-	-	5 (100)	0.18 ±0.04

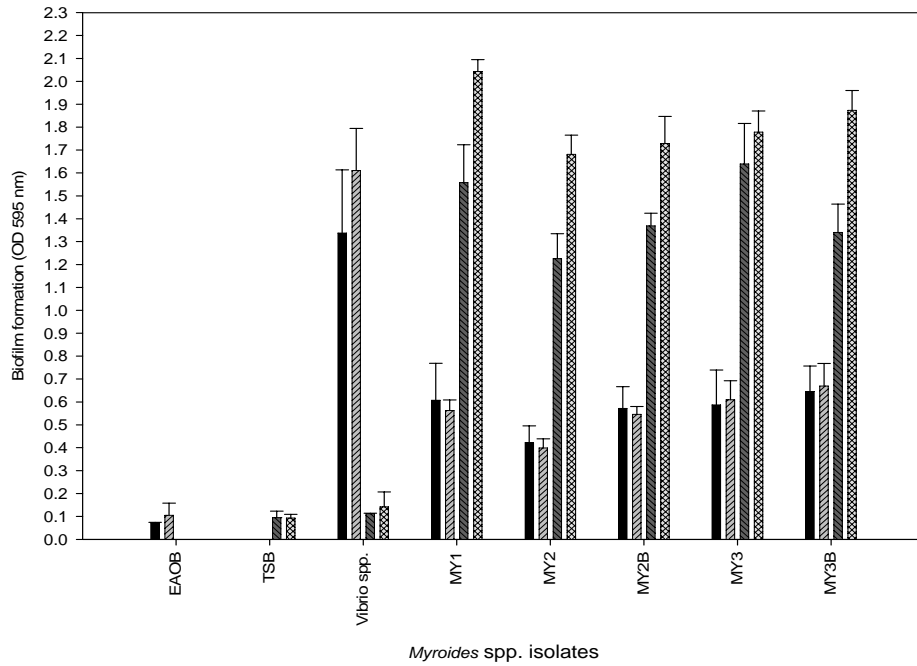


Figure 3. 1 Biofilm formation by *Myroides* and *Empedobacter* spp. study isolates on polystyrene microtitre plates, at room temperature in nutrient-rich media (TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

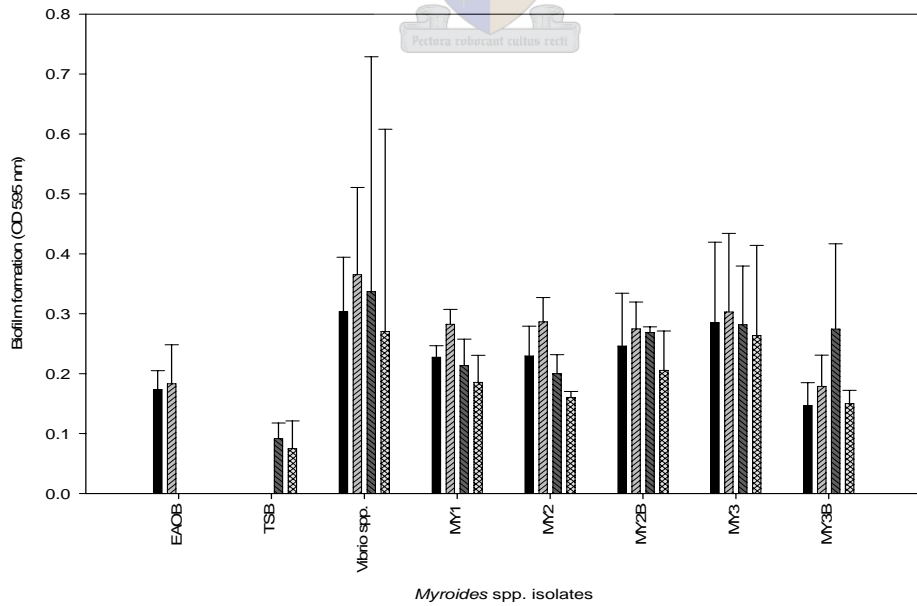


Figure 3. 2 Biofilm formation by *Myroides* and *Empedobacter* spp. study isolates on polystyrene microtitre plates, at 37 °C in nutrient-rich media (TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

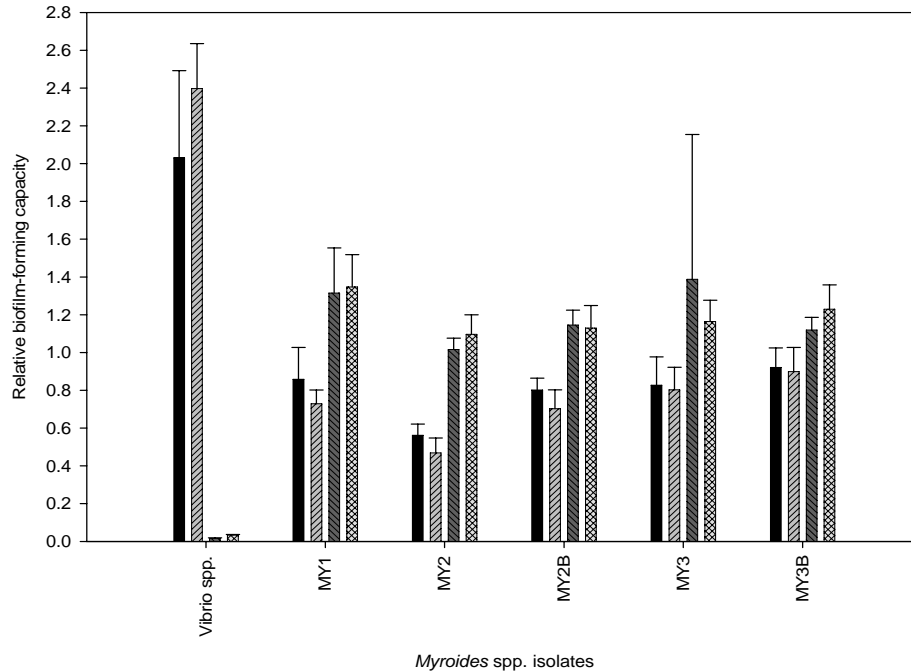


Figure 3.3 Relative biofilm-forming capacity of *Myroides* and *Empedobacter* spp. study isolates on polystyrene microtitre plates, at room temperature in nutrient-rich media (TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

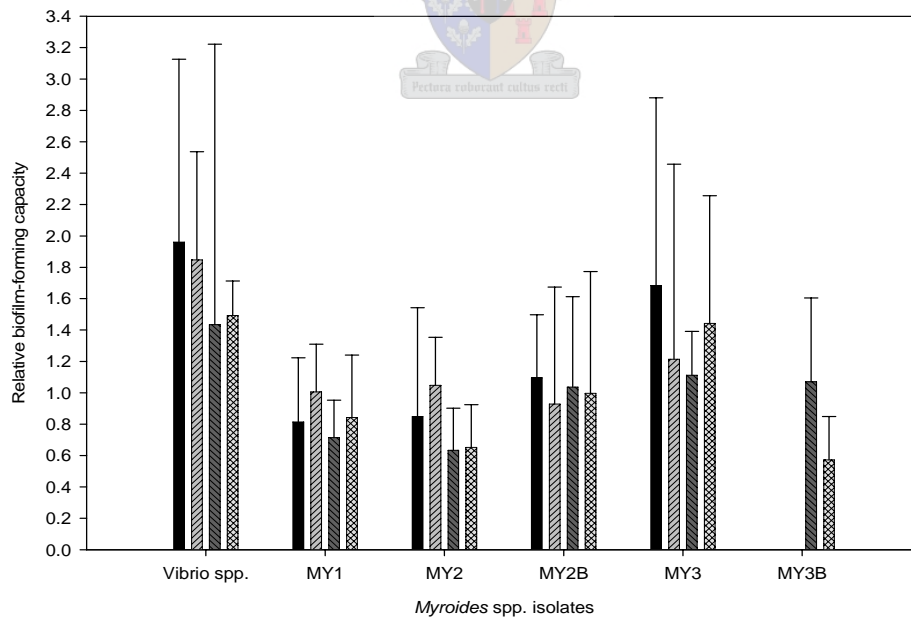


Figure 3.4 Relative biofilm-forming capacity of *Myroides* and *Empedobacter* spp. study isolates on polystyrene microtitre plates, at 37 °C in nutrient-rich media (TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

Initiation of adherence by the study isolates was assayed by incubating the microtitre plate for 16, 30 and 40 hours, under static and dynamic conditions at room temperature in nutrient-poor (EAOB) media. Adherence of the *Vibrio* spp. isolate increased over time with strongest adherence at 40 hours, regardless of static or dynamic conditions (Figs. 3.5-3.6). The *Myroides* and *Empedobacter* spp. isolates displayed strongest adherence within the first 16 hours, after which adherence appeared to decrease over time to 40 hours, regardless of agitation conditions (Figs. 3.5-3.6).

For the *Chryseobacterium* and *Elizabethkingia* spp. assays conducted at room temperature, biofilm formation and, therefore, relative biofilm-forming capacity ≤ 0.1 , respectively, was considered a negative result (in the case of 37 °C, biofilm-forming capacity ≤ 0.2 was considered a negative result). Biofilm formation values of the *Chryseobacterium* and *Elizabethkingia* spp. isolates at room temperature ranged from 0.07 (CH10) to 0.71 (CH25) in EAOB under dynamic conditions while under static conditions, biofilm formation ranged from 0.06 (CH22) to 0.83 (CH34) (Table 3.4; Fig. 3.7). At 37 °C, biofilm formation ranged from 0.11 (CH8, CH9) to 0.41 (CH29) under dynamic conditions in EAOB, while under static conditions biofilm formation ranged from 0.15 (CH27) to 0.65 (CH19) (Fig. 3.8). For TSB at room temperature under dynamic conditions, biofilm formation ranged from 0.07 (CH24) to 0.59 (CH13), while under static conditions biofilm formation ranged from 0.09 (CH24) to 0.80 (CH2B) (Table 3.4; Fig. 3.7). At 37 °C, biofilm formation in TSB under dynamic conditions ranged from 0.07 (CH15, CH16, CH17, CH19) to 0.84 (CH2B), while biofilm formation under static conditions ranged from 0.07 (CH1, CH16, CH18, CH19) to 0.50 (CH2B) (Fig 3.8).

In nutrient-poor media, 79.41 to 91.14% of the isolates were able to form biofilms at room temperature compared to 47.06 – 64.74% in nutrient-poor media at 37 °C under dynamic and static conditions, respectively (Table 3.5). All of the isolates formed biofilms at room temperature in nutrient-rich TSB medium under static conditions compared to 88.24% at 37 °C in TSB (Table 3.5). *Chryseobacterium* and *Elizabethkingia* spp. isolates therefore appeared to prefer nutrient-rich conditions at room temperature for biofilm formation. The relative biofilm-forming capacity of the study isolates was poor

at 37 °C (Fig. 3.10) compared to room temperature (Fig. 3.9), however, it was observed that study isolate CH2B formed biofilms best at 37 °C in TSB (Table 3.4; Fig. 3.10).

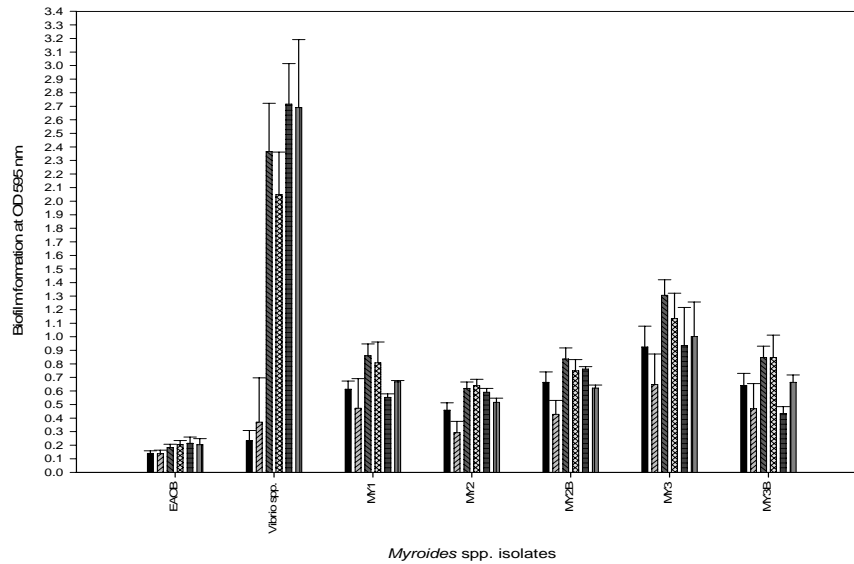


Figure 3.5 Time course of adherence exhibited by *Myroides* and *Empedobacter* spp. isolates under dynamic conditions in EAOB at room temperature for incubation times of 16, 24, 30 and 40 hours. 16 h dynamic (first column), 16 h static (second column), 30 h dynamic (third column), 30 h static (fourth column), 40 h dynamic (fifth column), and 40 h (sixth column).

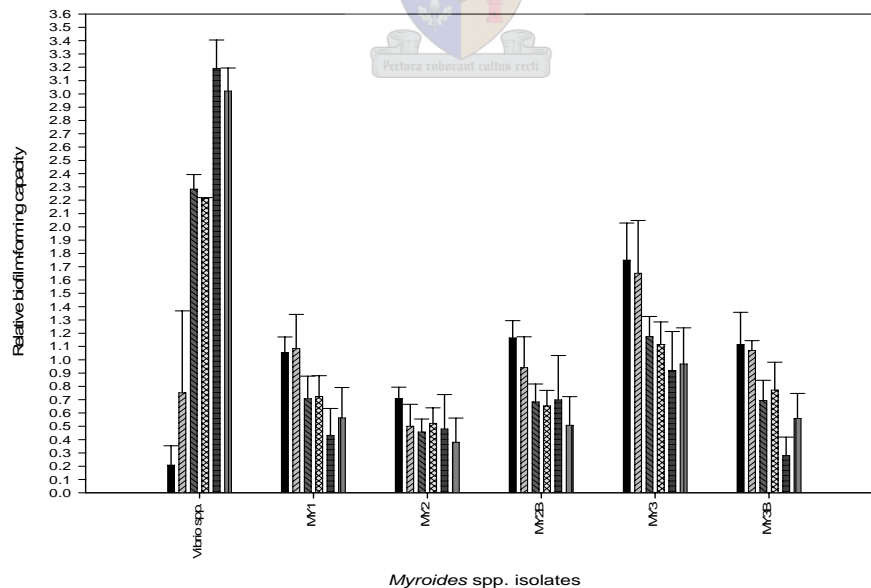


Figure 3.6 Time course of adherence exhibited by *Myroides* and *Empedobacter* spp. isolates under static conditions in EAOB at room temperature for incubation times of 16, 24, 30 and 40 hours. 16 h dynamic (first column), 16 h static (second column), 30 h dynamic (third column), 30 h static (fourth column), 40 h dynamic (fifth column), and 40 h (sixth column).

Significant differences ($p < 0.05$) in biofilm formation under static/dynamic conditions were observed at room temperature/37 °C in nutrient-rich and nutrient-poor media (EAOB room temperature- $p = 0.000$; 37 °C $p = 0.0001$; and TSB room temperature- $p = 0.000$; 37 °C $p = 0.000$). The *Vibrio* spp. isolate, and 9/32 (28.1 %) of the biofilm forming *Chryseobacterium* and *Elizabethkingia* spp. isolates preferred EAOB to TSB while 12/32 (37.5 %) of the isolates preferred TSB to EAOB.

Table 3.4 Biofilm formation values and profiles, and relative biofilm-formation capacity of *Chryseobacterium* and *Elizabethkingia* spp. study isolates following incubation at room temperature (~21 °C) under static or dynamic conditions in nutrient-rich (TSB) or nutrient-poor (EAOB) media, respectively.

Isolate	Biofilm formation [‡] (OD ₆₀₀) (Room temperature)								Relative biofilm-forming capacity [#] (Room temperature)							
	EAOB				TSB				EAOB				TSB			
	Average (OD±SD)		Average (OD±SD)		Average (OD±SD)		Average (OD±SD)		Average (OD±SD)		Average (OD±SD)		Average (OD±SD)			
	Dynamic	Static	Dynamic	Static	Dynamic	Static	Dynamic	Static	Dynamic	Static	Dynamic	Static	Dynamic	Static		
<i>Vibrio</i> spp.	1.34 ±0.28	S*	1.61 ±0.18	S	0.11 ±0.00	W	0.14 ±0.07	W	2.03 ±0.46	+	2.40 ±0.24	+	0.02 ±0.00	-	0.03 ±0.00	-
CH1	0.08 ±0.00	W	0.08 ±0.01	N	0.18 ±0.05	W	0.11 ±0.02	W	0.04 ±0.03	-	0.00 ±0.05	-	0.64 ±0.42	+	0.10 ±0.12	+
CH1B	0.12 ±0.02	W	0.18 ±0.03	W	0.19 ±0.05	W	0.20 ±0.05	M	0.27 ±0.19	+	0.48 ±0.20	+	0.68 ±0.35	+	0.58 ±0.26	+
CH2	0.13 ±0.04	W	0.18 ±0.07	W	0.19 ±0.04	W	0.19 ±0.04	M	0.35 ±0.26	+	0.46 ±0.53	+	0.68 ±0.26	+	0.51 ±0.53	+
CH2B	0.23 ±0.02	M	0.31 ±0.13	M	0.44 ±0.13	S	0.80 ±0.06	S	0.92 ±0.22	+	1.22 ±1.08	+	2.56 ±1.53	+	3.84 ±1.95	+
CH3	0.16 ±0.04	M	0.21 ±0.05	M	0.25 ±0.03	M	0.26 ±0.06	M	0.49 ±0.33	+	0.66 ±0.36	+	1.11 ±0.17	+	0.90 ±0.45	+
CH4	0.12 ±0.02	W	0.19 ±0.02	W	0.20 ±0.03	M	0.23 ±0.02	M	0.28 ±0.14	+	0.54 ±0.16	+	0.78 ±0.21	+	0.73 ±0.20	+
CH4B	0.13 ±0.03	W	0.17 ±0.06	W	0.22 ±0.04	M	0.21 ±0.06	M	0.32 ±0.23	+	0.36 ±0.44	+	0.95 ±0.31	+	0.66 ±0.58	+
CH5	0.14 ±0.03	W	0.16 ±0.06	W	0.21 ±0.04	M	0.18 ±0.02	W	0.36 ±0.26	+	0.34 ±0.44	+	0.88 ±0.40	+	0.49 ±0.46	+
CH6	0.14 ±0.03	W	0.19 ±0.03	W	0.20 ±0.08	M	0.19 ±0.03	M	0.40 ±0.23	+	0.49 ±0.24	+	0.78 ±0.79	+	0.54 ±0.23	+
CH7	0.11 ±0.03	W	0.19 ±0.05	W	0.24 ±0.04	M	0.23 ±0.06	M	0.24 ±0.24	+	0.51 ±0.36	+	1.05 ±0.41	+	0.74 ±0.28	+
CH8	0.12 ±0.01	W	0.16 ±0.06	W	0.20 ±0.03	M	0.18 ±0.03	W	0.28 ±0.07	+	0.35 ±0.42	+	0.75 ±0.18	+	0.44 ±0.14	+
CH9	0.09 ±0.02	W	0.10 ±0.01	N	0.10 ±0.03	W	0.10 ±0.05	W	0.12 ±0.16	+	0.00 ±0.10	-	0.07 ±0.21	+	0.05 ±0.25	-
CH10	0.07 ±0.00	N	0.07 ±0.01	N	0.48 ±0.21	S	0.62 ±0.13	S	0.00 ±0.02	-	0.00 ±0.06	-	2.81 ±1.94	+	2.85 ±1.61	+
CH11	0.36 ±0.03	S	0.37 ±0.03	M	0.23 ±0.01	M	0.28 ±0.06	M	1.65 ±0.10	+	1.63 ±0.31	+	0.97 ±0.12	+	1.01 ±0.45	+
CH12	0.35 ±0.03	S	0.39 ±0.07	M	0.26 ±0.05	M	0.24 ±0.04	M	1.61 ±0.20	+	1.72 ±0.68	+	1.24 ±0.29	+	0.82 ±0.34	+
CH13	0.31 ±0.14	S	0.26 ±0.07	M	0.59 ±0.08	S	0.69 ±0.05	S	1.39 ±0.78	+	0.92 ±0.59	+	3.63 ±0.67	+	3.24 ±0.64	+
CH14	0.45 ±0.22	S	0.43 ±0.03	S	0.57 ±0.08	S	0.53 ±0.06	S	2.19 ±0.77	+	1.99 ±0.28	+	3.54 ±0.46	+	2.39 ±0.70	+
CH15	0.12 ±0.01	W	0.12 ±0.02	W	0.25 ±0.05	M	0.28 ±0.08	M	0.29 ±0.08	+	0.11 ±0.21	+	1.13 ±0.48	+	0.99 ±0.58	+
CH16	0.12 ±0.02	W	0.13 ±0.01	W	0.25 ±0.05	M	0.27 ±0.05	M	0.29 ±0.14	+	0.14 ±0.09	+	1.14 ±0.44	+	0.96 ±0.40	+
CH17	0.15 ±0.01	M	0.14 ±0.04	W	0.26 ±0.06	M	0.28 ±0.04	M	0.44 ±0.07	+	0.20 ±0.33	+	1.19 ±0.46	+	1.03 ±0.42	+
CH18	0.14 ±0.02	W	0.15 ±0.03	W	0.27 ±0.04	M	0.30 ±0.05	M	0.37 ±0.18	+	0.29 ±0.20	+	1.30 ±0.35	+	1.12 ±0.38	+
CH19	0.14 ±0.01	W	0.18 ±0.04	W	0.26 ±0.03	M	0.24 ±0.06	M	0.40 ±0.12	+	0.48 ±0.28	+	1.20 ±0.31	+	0.81 ±0.44	+
CH21	0.10 ±0.02	W	0.16 ±0.10	W	0.14 ±0.01	W	0.13 ±0.04	W	0.18 ±0.15	+	0.36 ±0.72	+	0.33 ±0.10	+	0.22 ±0.25	+
CH22	0.08 ±0.02	W	0.06 ±0.01	N	0.08 ±0.00	N	0.14 ±0.06	W	0.07 ±0.11	-	0.00 ±0.08	-	0.00 ±0.23	-	0.28 ±0.36	+
CH23	0.07 ±0.01	N	0.06 ±0.01	N	0.09 ±0.02	N	0.11 ±0.05	W	0.00 ±0.04	-	0.00 ±0.10	-	0.00 ±0.17	-	0.11 ±0.30	-
CH24	0.07 ±0.00	N	0.07 ±0.00	N	0.07 ±0.00	N	0.09 ±0.03	W	0.00 ±0.03	-	0.00 ±0.03	-	0.00 ±0.03	-	0.00 ±0.18	-
CH25	0.71 ±0.16	S	0.69 ±0.06	S	0.13 ±0.05	W	0.18 ±0.08	W	3.69 ±0.69	+	3.52 ±0.66	+	0.29 ±0.37	+	0.49 ±0.43	+
CH26	0.20 ±0.05	M	0.20 ±0.02	W	0.11 ±0.02	W	0.19 ±0.07	M	0.74 ±0.28	+	0.55 ±0.18	+	0.12 ±0.12	+	0.53 ±0.37	+
CH27	0.09 ±0.02	W	0.07 ±0.01	N	0.10 ±0.04	W	0.13 ±0.07	W	0.12 ±0.17	+	0.00 ±0.11	-	0.04 ±0.26	-	0.21 ±0.37	+
CH28	0.35 ±0.08	S	0.40 ±0.11	M	0.13 ±0.04	W	0.18 ±0.05	W	1.57 ±0.11	+	1.75 ±0.92	+	0.25 ±0.24	+	0.50 ±0.31	+
CH29	0.42 ±0.10	S	0.37 ±0.07	M	0.21 ±0.04	M	0.37 ±0.19	M	1.98 ±0.48	+	1.61 ±0.49	+	0.85 ±0.33	+	1.52 ±1.05	+
CH30	0.38 ±0.07	S	0.39 ±0.04	M	0.21 ±0.04	M	0.64 ±0.03	S	1.77 ±0.29	+	1.71 ±0.43	+	0.87 ±0.33	+	2.95 ±1.86	+
CH33	0.38 ±0.03	S	0.17 ±0.04	W	0.14 ±0.04	W	0.20 ±0.04	M	1.78 ±0.31	+	0.38 ±0.27	+	0.30 ±0.34	+	0.60 ±0.20	+
CH34	0.63 ±0.15	S	0.83 ±0.06	S	0.51 ±0.06	S	0.56 ±0.04	S	3.24 ±0.20	+	4.41 ±0.18	+	3.04 ±0.35	+	2.53 ±0.20	+

[‡] Biofilm formation assayed according to Stepanovic *et al.* (2000).

* W/M/S refers to adherence categories displayed by isolates according to criteria described by Stepanovic *et al.* (2000).

[#] Relative biofilm-forming capacity described by Van Houdt *et al.* (2004).

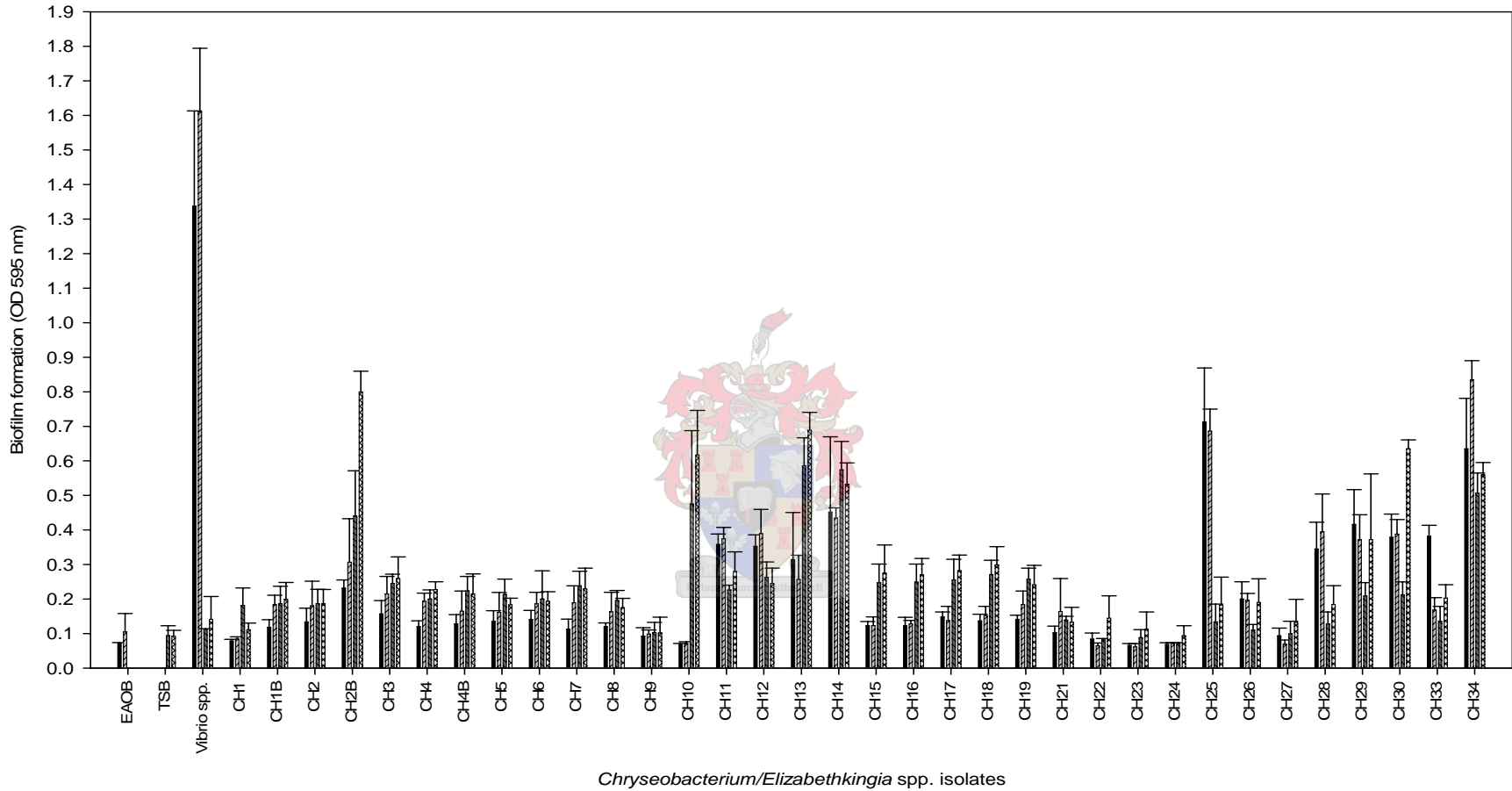


Figure 3. 7

Biofilm formation by *Chryseobacterium* and *Elizabethkingia* spp. study isolates on polystyrene microtitre plates, at room temperature in nutrient-rich media (TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

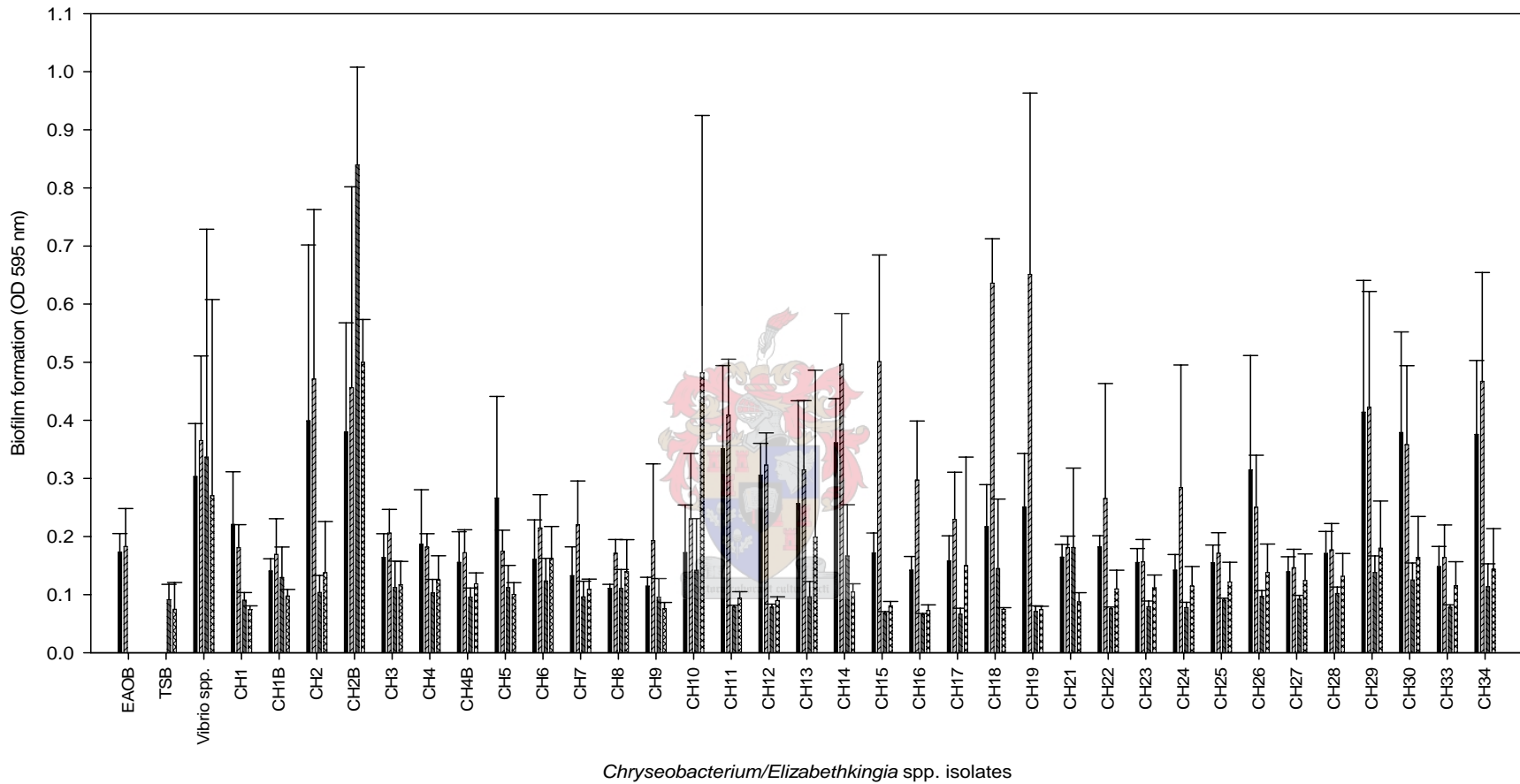


Figure 3.8 Biofilm formation by *Chryseobacterium* and *Elizabethkingia* spp. study isolates on polystyrene microtitre plates, at 37 °C in nutrient-rich media(TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

Table 3. 5 Biofilm formation by *Chryseobacterium* and *Elizabethkingia* spp. study isolates (n = 34) following incubation at room temperature (~21 °C) and 37 °C, under static or dynamic conditions in nutrient-rich (TSB) or nutrient-poor (EAOB) media, respectively.

Parameters	Number of isolates											
	Non-adherent		Biofilm formation								Total	
	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD		
21 °C EAOB dynamic	3 (8.82)	0.07 ±0.00	17 (50)	0.12 ±0.02	4 (11.76)	0.18 ±0.04	10 (29.41)	0.43 ±0.13	31 (91.18)	0.23 ±0.16		
21 °C EAOB static	7 (20.59)	0.07 ±0.01	16 (47.06)	0.17 ±0.02	8 (23.53)	0.34 ±0.07	3 (8.82)	0.65 ±0.20	27 (79.41)	0.27 ±0.17		
21 °C TSB dynamic	3 (8.82)	0.08 ±0.01	10 (29.41)	0.14 ±0.03	16 (47.06)	0.23 ±0.02	5 (14.71)	0.52 ±0.06	31 (91.18)	0.25 ±0.13		
21 °C TSB static	-	-	11 (32.35)	0.14 ±0.03	17 (50)	0.25 ±0.05	6 (17.65)	0.64 ±0.10	34 (100)	0.28 ±0.18		
37 °C EAOB dynamic	18 (52.94)	0.15 ±0.02	9 (26.47)	0.24 ±0.05	7 (20.59)	0.38 ±0.02	-	-	16 (47.06)	0.31 ±0.10		
37 °C EAOB static	12 (35.29)	0.17 ±0.01	13 (38.24)	0.26 ±0.05	9 (26.47)	0.50 ±0.09	-	-	22 (64.74)	0.38 ±0.17		
37 °C TSB dynamic	12 (35.29)	0.08 ±0.01	21 (61.76)	0.12 ±0.02	-	-	1 (2.94)	0.84 ±0.00	22 (64.71)	0.48 ±0.51		
37 °C TSB static	4 (11.76)	0.07 ±0.00	24 (70.59)	0.11 ±0.02	4 (11.76)	0.18 ±0.02	2 (5.88)	0.49 ±0.01	30 (88.24)	0.26 ±0.20		

Incubation time variation indicated that adherence of the *Vibrio* spp. isolate increased over time with strongest adherence at 40 hours, regardless of agitation conditions (Figs. 3.11-3.12). All of the *Chryseobacterium* and *Elizabethkingia* spp. isolates displayed biofilm formation after 16 h of incubation under dynamic conditions (Table 3.6; Fig. 3.11). Contrary to that observed for the *Vibrio* spp. isolate, biofilm formation by the study isolates generally decreased as incubation time increased with only 47.06% of the isolates capable of biofilm-formation after 40 h of incubation (Table 3.6; Fig 3.11).

Table 3. 6 Biofilm formation by *Chryseobacterium* and *Elizabethkingia* spp. study isolates (n = 34) following incubation for 16 h, 30 h, and 40 h at room temperature (~21 °C), under static or dynamic conditions in nutrient-poor (EAOB) media, respectively.

Parameters	Number of isolates											
	Non-adherent		Biofilm formation								Total	
	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD	No. (%)	Average OD±SD		
16 hours dynamic	-	-	13 (38.24)	0.20 ±0.05	16 (47.06)	0.35 ±0.06	5 (14.71)	0.83 ±0.10	34 (100)	0.37 ±0.22		
16 hours static	7 (20.59)	0.12 ±0.01	14 (41.18)	0.23 ±0.04	8 (23.53)	0.32 ±0.08	5 (14.71)	0.70 ±0.08	27 (79.41)	0.34 ±0.19		
30 hours dynamic	1 (2.94)	0.18 ±0.00	25 (73.53)	0.24 ±0.04	5 (14.71)	0.43 ±0.04	3 (8.82)	0.87 ±0.02	33 (97.06)	0.33 ±0.19		
30 hours static	9 (26.47)	0.19 ±0.01	19 (55.88)	0.28 ±0.06	4 (11.76)	0.58 ±0.16	2 (5.88)	1.00 ±0.12	25 (73.53)	0.39 ±0.23		
40 hours dynamic	18 (52.94)	0.18 ±0.01	11 (32.35)	0.28 ±0.07	4 (11.76)	0.63 ±0.16	1 (2.94)	0.92 ±0.00	16 (47.06)	0.41 ±0.23		
40 hours static	14 (41.18)	0.17 ±0.01	14 (41.18)	0.27 ±0.04	6 (17.65)	0.67 ±0.13	-	-	20 (58.82)	0.39 ±0.20		

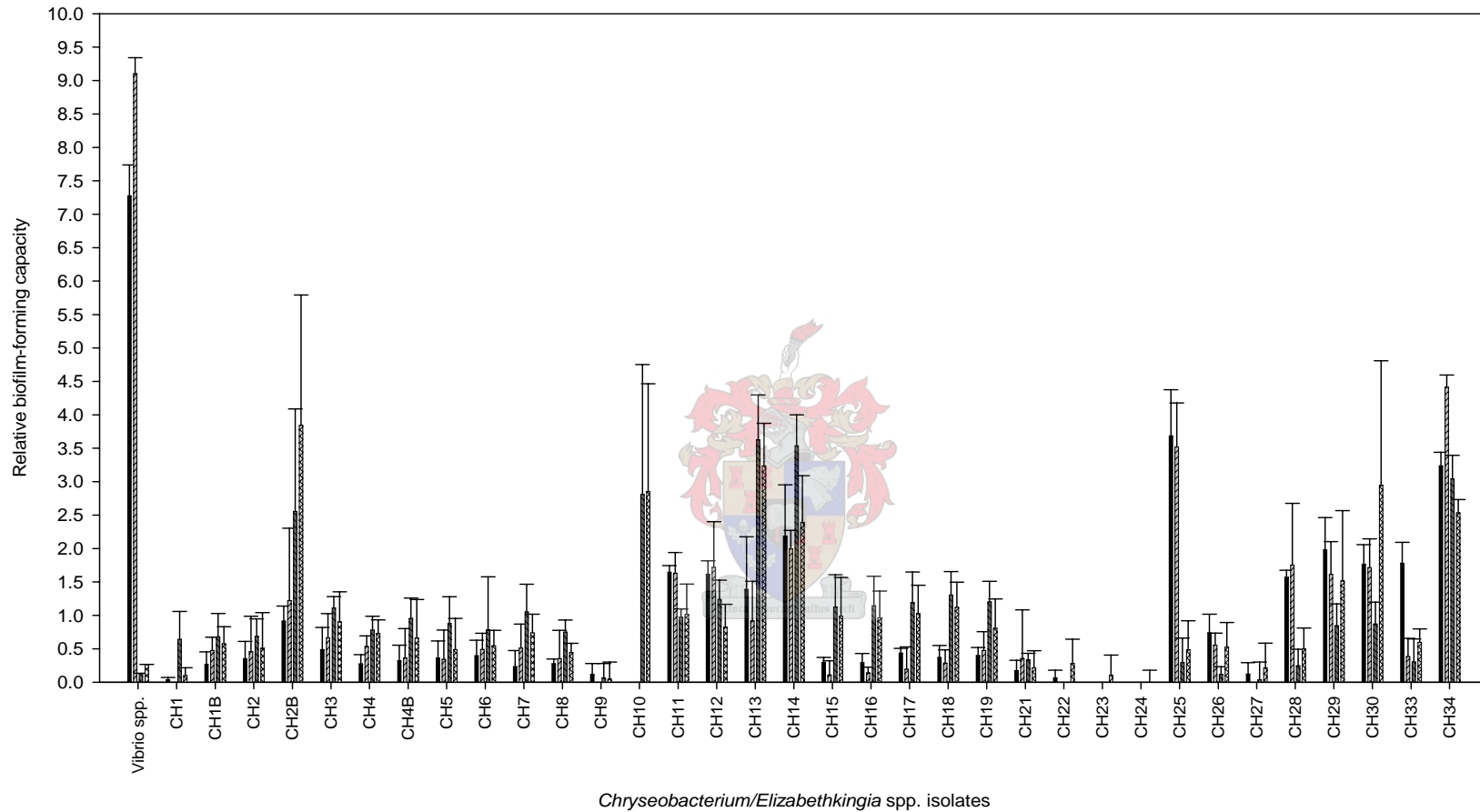


Figure 3. 7 Relative biofilm-forming capacity of *Chryseobacterium* and *Elizabethkingia* spp. study isolates on polystyrene microtitre plates, at room temperature in nutrient-rich media (TSB) and nutrient-poor media (EAOB) under dynamic and static conditions. EAOB dynamic (first column), EAOB static (second column), TSB dynamic (third column), TSB static (fourth column).

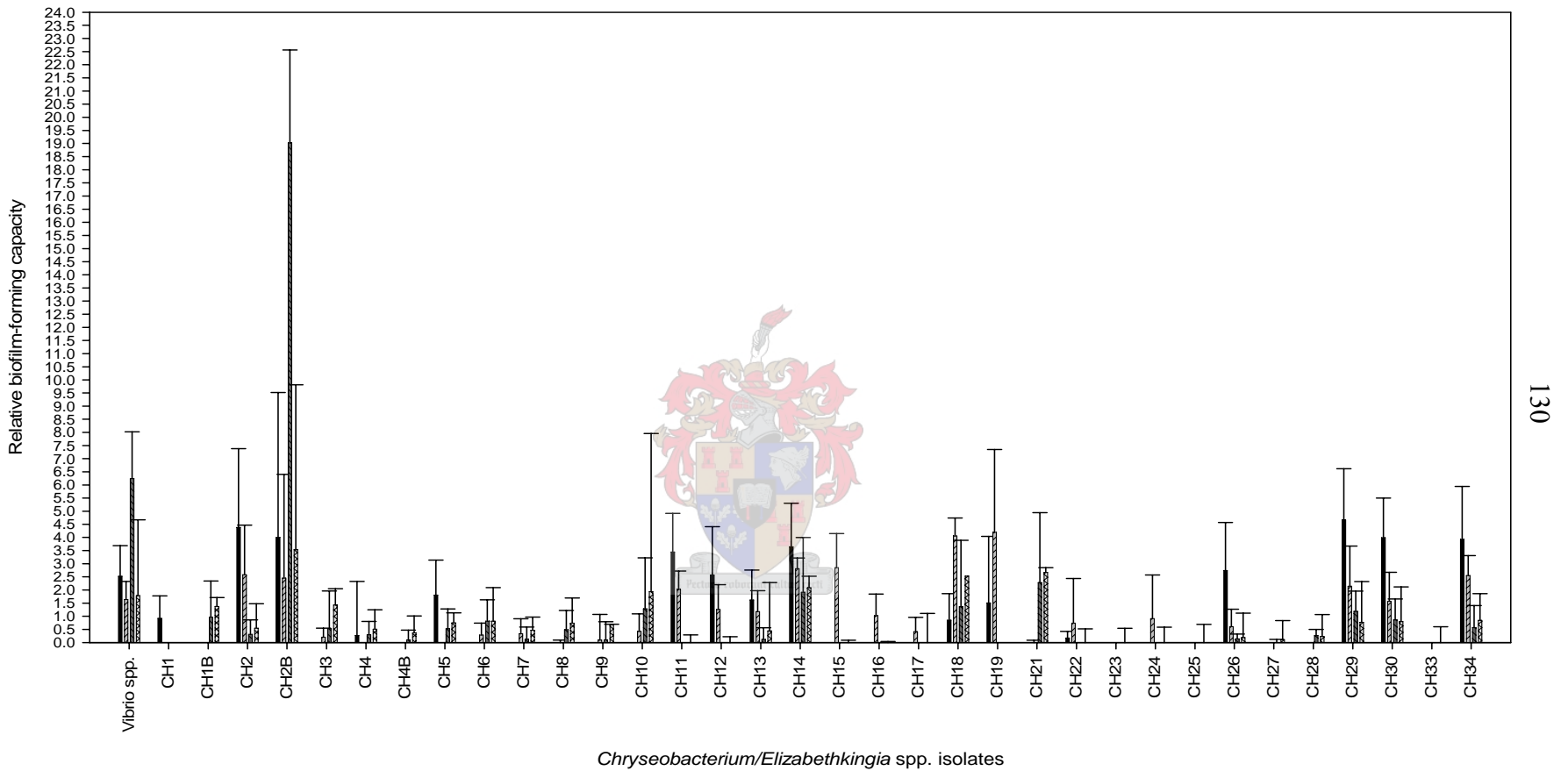


Figure 3.8 Relative biofilm-forming capacity of *Chryseobacterium* and *Elizabethkingia* spp. study isolates on polystyrene microtitre plates, at 37 °C in nutrient-rich media (TSB) and nutrient-poor media (EAOb) under dynamic and static conditions. EAOb dynamic (first column), EAOb static (second column), TSB dynamic (third column), TSB static (fourth column).

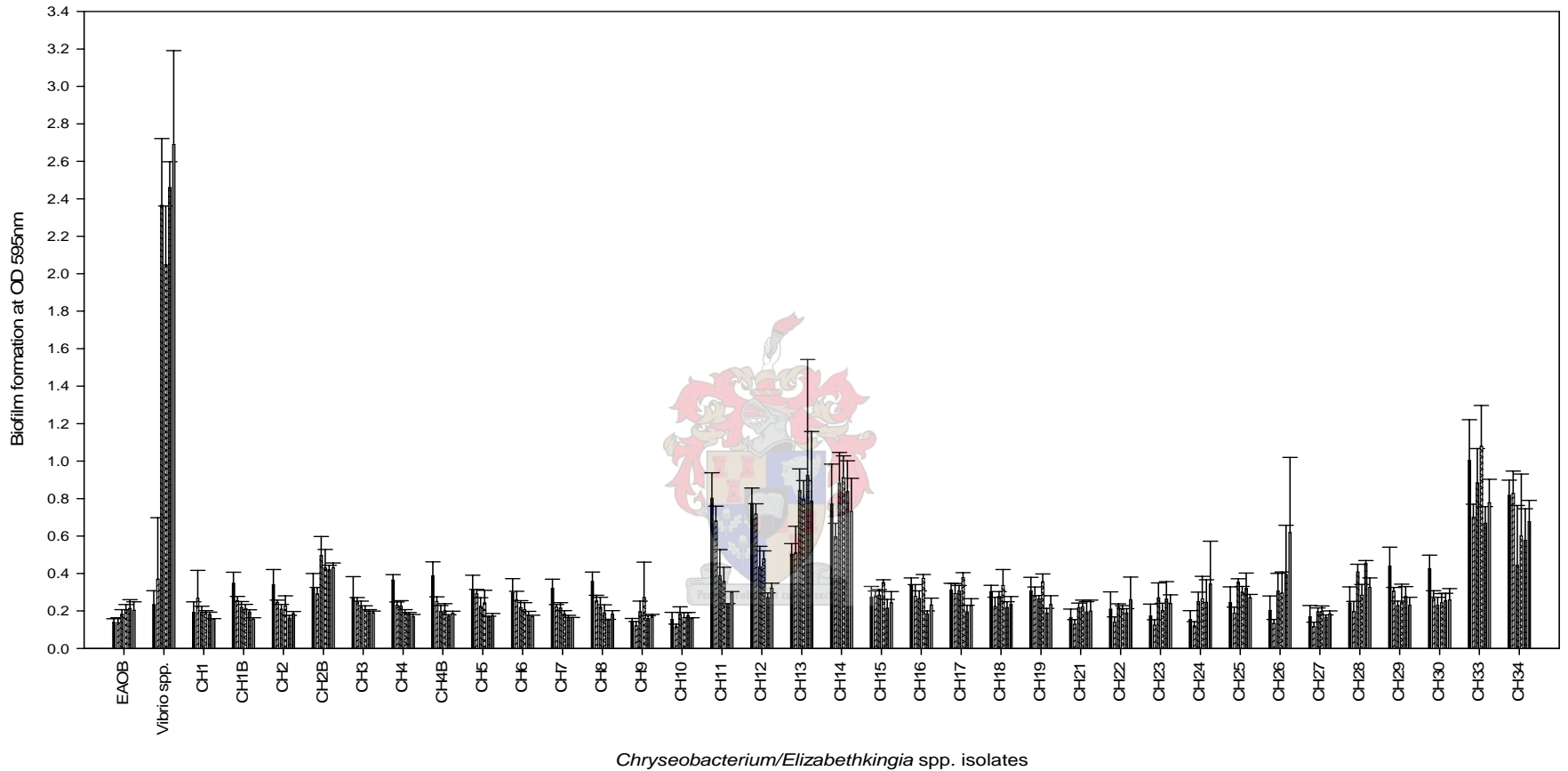


Figure 3.11

Time course of biofilm formation by *Chryseobacterium* and *Elizabethkingia* spp. isolates at 16, 30 and 40 hours of incubation, under dynamic and static conditions in EAOB at room temperature. 16 h dynamic (first column), 16 h static (second column), 30 h dynamic (third column), 30 h static (fourth column), 40 h dynamic (fifth column), 40 h static (sixth column).

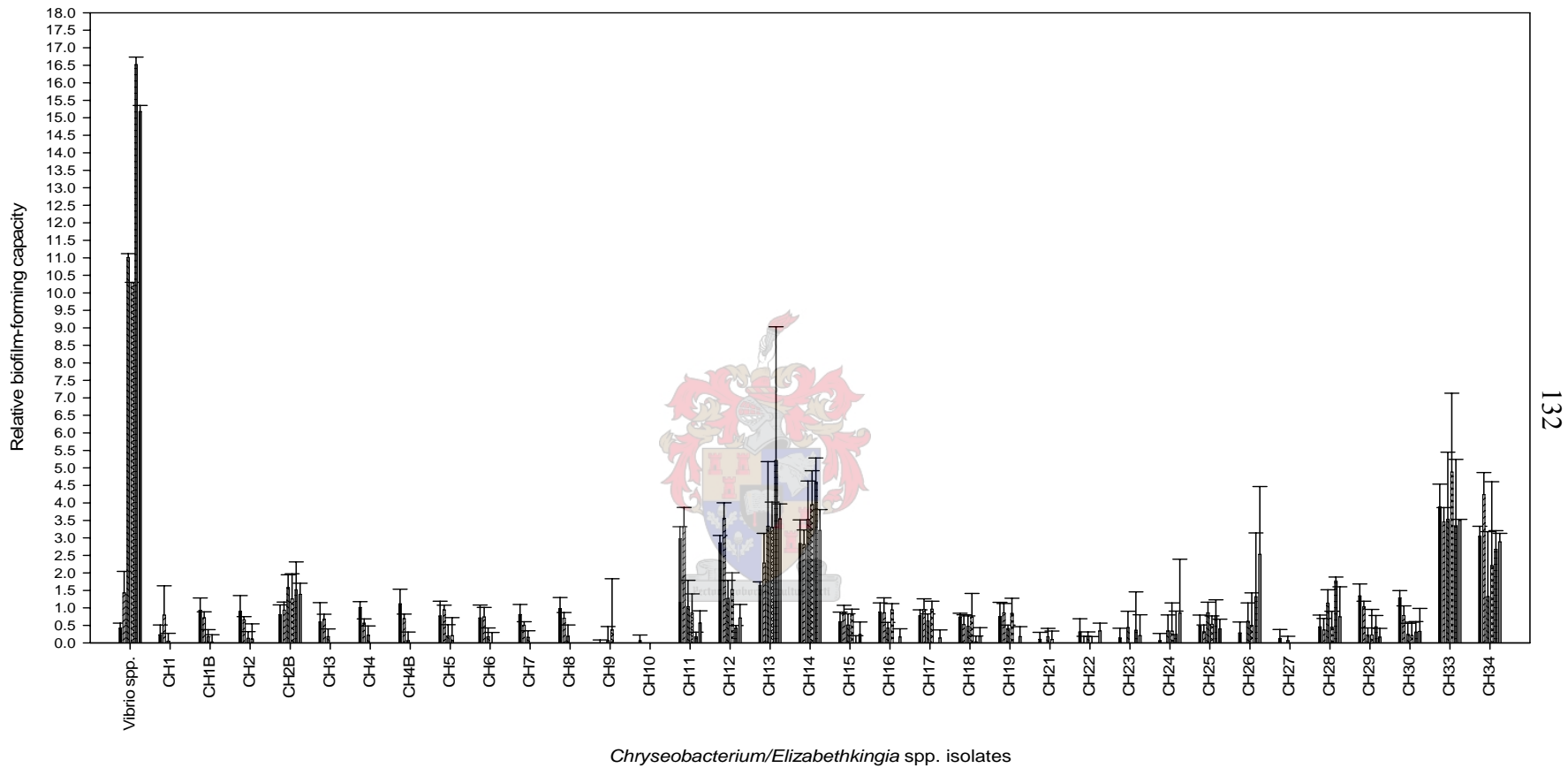


Figure 3. 12

Time course of biofilm-forming capacity of *Chryseobacterium* and *Elizabethkingia* spp. isolates at 16, 30 and 40 hours of incubation, under dynamic and static conditions in EA0B at room temperature. 16 h dynamic (first column), 16 h static (second column), 30 h dynamic (third column), 30 h static (fourth column), 40 h dynamic (fifth column), 40 h static (sixth column).

3.3.1.1 Induction experiments

Induction experiments were carried out in nutrient-rich media (TSB) at room temperature under static conditions. *Myroides* / *Empedobacter* spp. isolate MY1 displayed strong adherence under these conditions with a biofilm-forming capacity (BFC) value of 2.29 (Table 3.7; Fig. 3.13).

Although increases in biofilm formation was observed in the spent supernatant of *A. hydrophila* (BFC - 3.62), *A. salmonicida* (BFC - 3.19), *A. sobria* (BFC - 7.69) and *S. enterica* serovar *Arizonae* (BFC - 6.11), none of the increases were found to be significantly higher than that obtained in TSB without added spent supernatants (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) (Table 3.7; Fig. 3.13). Similarly, the *Chryseobacterium* / *Elizabethkingia* spp. isolates CH23 and CH26 displaying strong adherence under experimental conditions with biofilm-forming capacities of 1.94 and 2.21, respectively, did not display significant induction of adherence in the presence of spent supernatants (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) (Table 3.7; Fig. 3.13). Study isolate CH2B displayed moderate adherence under experimental conditions with a biofilm-forming capacity value of 0.69. Significant increases in adherence by this study isolate was observed in the presence of spent supernatants from *E. coli* (BFC - 1.04; $p = 0.0019$), *L. monocytogenes* (BFC - 1.73; $p = 0.0004$), *S. enterica* serovar *Arizonae* (BFC - 0.97; $p = 0.0022$), and *F. johnsoniae*-like spp. YO59 (BFC - 0.90; $p = 0.0005$) (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) (Table 3.7; Fig. 3.13).

Study isolate CH34 also displayed moderate adherence with a biofilm-forming capacity of 0.71 which was significantly increased with the spent supernatant of *Chryseobacterium* / *Elizabethkingia* spp. isolate CH2B (BFC - 2.47; $p = 0.0002$) (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) (Table 3.7; Fig. 3.13). Weakly adherent *Chryseobacterium* / *Elizabethkingia* spp. study isolates CH15 (BFC - 0.13) and CH25 (BFC - 0.04) were induced to a significantly stronger adherence phenotype by 8/12 (67 %) and 7/12 (58 %) of the inducing spent supernatants, respectively, of which *A. hydrophila*, *P. aeruginosa*, *F. johnsoniae*-like spp. YO59, and the *Vibrio* spp. isolate, caused significant induction in both study isolates (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) (Table 3.7; Fig. 3.13). Study isolate CH8 showed a non-biofilm-forming

phenotype in TSB at room temperature under static conditions. A biofilm-forming phenotype was induced by spent supernatants of *A. salmonicida* (BFC – 1.35; $p = 0.0000$), *E. coli* (BFC – 0.94; $p = 0.0000$), *E. tarda* (BFC – 1.49; $p = 0.0030$), *L. monocytogenes* (BFC – 0.35; $p = 0.0004$), *P. aeruginosa* (BFC – 1.17; $p = 0.0009$), and the *Vibrio* spp. isolates (BFC – 0.32; $p = 0.0000$) (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) (Table 3.7; Fig. 3.13). From these results it was evident that the study isolates were not induced to a stronger adherence phenotype when an already strongly adherence profile was recorded in TSB without added spent supernatants.

Table 3.7 Relative biofilm-formation capacity of the *Myroides* and *Empedobacter* spp. isolate (MY1) and selected *Chryseobacterium* and *Elizabethkingia* spp. study isolates following exposure to spent medium of 13 Gram-negative and Gram-positive bacteria at room temperature (~21 °C) under static conditions in nutrient-rich (TSB) media.

Test isolates	<i>Myroides</i> / <i>Empedobacter</i> spp. isolate and <i>Chryseobacterium</i> / <i>Elizabethkingia</i> spp. isolates relative biofilm-forming capacity							
	MY1	CH2B	CH8	CH15	CH23	CH25	CH26	CH34
Nutrient-rich media (TSB)	2.29 ±0.17	0.69 ±0.17	0.00 ±0.00	0.13 ±0.06	1.94 ±0.57	0.04 ±0.03	2.21 ±0.50	0.71 ±0.15
<i>A. hydrophila</i>	3.62 ±4.05	0.45 ±0.79	0.00 ±0.00	0.41 ±0.70	0.27 ±0.47	0.07 ±0.13	0.00 ±0.00	0.50 ±0.87
<i>A. salmonicida</i>	3.19 ±2.90	0.56 ±0.98	1.34 ±0.67	0.46 ±0.54	1.04 ±0.79	0.00 ±0.00	1.04 ±1.44	0.34 ±0.58
<i>A. sobria</i>	7.69 ±0.53	0.00 ±0.00	0.00 ±0.00	0.31 ±0.53	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
<i>Chryseobacterium</i> / <i>Elizabethkingia</i> spp. isolate CH2B	1.05 ±0.59	1.02 ±1.11	0.00 ±0.17	2.39 ±0.96	1.30 ±0.87	0.00 ±0.48	0.20 ±0.84	2.47 ±0.28
<i>E. coli</i>	1.53 ±0.29	1.03 ±0.10	0.94 ±0.08	1.11 ±0.34	1.73 ±0.56	0.00 ±0.06	0.88 ±0.05	0.85 ±0.33
<i>E. tarda</i>	2.10 ±0.19	0.93 ±0.21	1.49 ±0.70	1.08 ±0.45	0.39 ±0.52	0.78 ±0.19	0.48 ±0.06	0.74 ±0.21
<i>L. innocua</i>	2.20 ±0.61	1.65 ±0.22	0.00 ±0.10	0.51 ±0.30	0.54 ±0.20	0.65 ±0.07	1.02 ±0.12	1.37 ±0.24
<i>L. monocytogenes</i>	1.14 ±0.14	1.73 ±0.19	0.35 ±0.12	1.67 ±0.13	1.70 ±0.14	0.33 ±0.13	0.36 ±0.17	0.72 ±0.07
<i>P. aeruginosa</i>	0.00 ±0.38	1.51 ±0.67	1.17 ±0.50	1.30 ±0.31	1.02 ±0.60	1.59 ±0.25	0.73 ±0.46	1.84 ±0.40
<i>S. enterica</i>	6.11 ±3.43	0.97 ±0.23	0.00 ±1.00	0.00 ±1.15	0.85 ±1.47	1.01 ±0.44	0.00 ±0.93	0.71 ±0.51
<i>Flavobacterium</i> spp. isolate YO59	1.99 ±0.26	0.90 ±0.10	0.29 ±0.27	1.05 ±0.29	0.95 ±0.32	0.81 ±0.05	0.81 ±0.11	1.19 ±0.25
<i>Vibrio</i> spp.	0.31 ±0.54	0.33 ±0.57	0.32 ±0.56	0.30 ±0.52	0.30 ±0.52	0.33 ±0.57	0.35 ±0.61	0.42 ±0.72

* Relative biofilm-forming capacity according to Van Houdt *et al.*, 2004

3.3.2 Cell-surface hydrophobicity assays

3.3.2.1 Bacterial adherence to hydrocarbons (BATH)

BATH values for the *Myroides* and *Empedobacter* spp. isolates ranged from 3.03 to 5.13 %, thus all of the isolates were classified as very hydrophilic (Table 3.8). It was not possible to correlate biofilm formation or biofilm-forming capacity of *Myroides* and *Empedobacter* spp. study isolates at room temperature/37 °C in TSB/EAOB with the BATH hydrophobicity measurements.

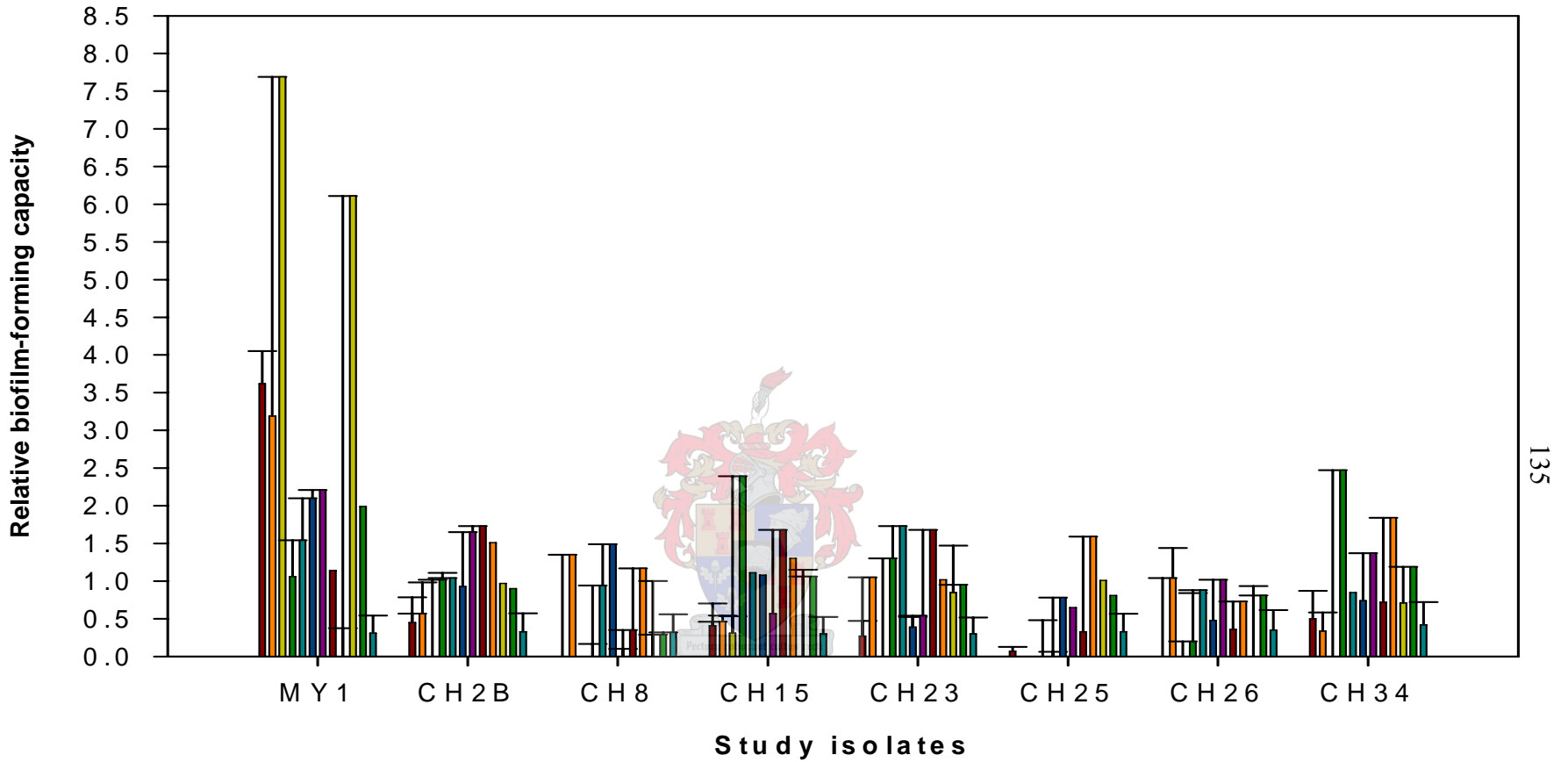


Figure 3.9 Relative biofilm-forming capacity of the *Myroides* and *Empedobacter* spp. isolate (MY1) and selected *Chryseobacterium* and *Elizabethkingia* spp. isolates following exposure to spent medium, at room temperature in nutrient-rich media (TSB) under static conditions. The filtered supernatants of 13 bacterial isolates were used as follows:

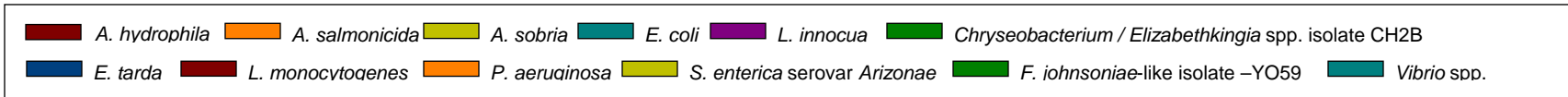


Table 3. 8 Cell surface hydrophobicity (BATH and SAT assays) values and profiles, capsule presence and Congo red accumulation results for *Myroides* and *Empedobacter* and *Chryseobacterium* and *Elizabethkingia* spp. isolates.

Isolate	Surface hydrophobicity				Capsule [†]	Congo Red
	BATH* (%)		SAT [#] (NH ₄) ₂ SO ₄			
	Ave±SD	Profile	Ave±SD	Profile		
<i>Myroides/Empedobacter</i> spp. isolates						
MY1	4.86 ±0.39	very hydrophilic	3 ±0	Very hydrophilic	++	+
MY2	3.16 ±1.20	very hydrophilic	3 ±0	Very hydrophilic	++	+
MY2B	5.05 ±0.21	very hydrophilic	3 ±0	Very hydrophilic	++	+
MY3	3.03 ±0.49	very hydrophilic	2.5 ±0.2	Very hydrophilic	++	+
MY3B	5.13 ±0.71	very hydrophilic	2.5 ±0	Very hydrophilic	++	+
<i>Chryseobacterium/Elizabethkingia</i> spp. isolates						
CH1	7.67 ±0.65	very hydrophilic	0.5 ±0	Moderate hydrophobicity	+	+
CH1B	4.67 ±0.70	very hydrophilic	0.5 ±0	Moderate hydrophobicity	+	+
CH2	5.40 ±0.78	very hydrophilic	0.2 ±0.08	Moderate hydrophobicity	+	+
CH2B	0.77 ±1.48	very hydrophilic	4 ±0	very hydrophilic	++	+
CH3	19.14 ±1.37	very hydrophilic	0.5 ±0.12	Moderate hydrophobicity	+	+
CH4	23.21 ±2.65	Moderate hydrophobicity	0.5 ±0	Moderate hydrophobicity	(+)	+
CH4B	16.84 ±0.76	very hydrophilic	0.5 ±0.12	Moderate hydrophobicity	(+)	+
CH5	17.15 ±0.28	very hydrophilic	0.5 ±0	Moderate hydrophobicity	+	+
CH6	11.73 ±1.21	very hydrophilic	0.5 ±0.12	Moderate hydrophobicity	+	+
CH7	18.93 ±4.76	very hydrophilic	0.5 ±0	Moderate hydrophobicity	+	+
CH8	14.17 ±0.63	very hydrophilic	0.5 ±0	Moderate hydrophobicity	(+)	+
CH9	28.12 ±3.45	Moderate hydrophobicity	2.5 ±0	very hydrophilic	++	+
CH10	11.71 ±1.06	very hydrophilic	1 ±0.26	Moderate hydrophobicity	-	+
CH11	24.99 ±2.97	Moderate hydrophobicity	3 ±0	very hydrophilic	(+)	+
CH12	17.25 ±2.21	very hydrophilic	3 ±0	very hydrophilic	-	+
CH13	12.66 ±0.39	very hydrophilic	1.5 ±0	very hydrophilic	(+)	+
CH14	15.09 ±3.97	very hydrophilic	1.5 ±0	very hydrophilic	(+)	+
CH15	40.90 ±1.39	Moderate hydrophobicity	1.5 ±0	very hydrophilic	(+)	+
CH16	33.18 ±5.03	Moderate hydrophobicity	1.5 ±0	very hydrophilic	(+)	+
CH17	30.70 ±4.41	Moderate hydrophobicity	1.5 ±0	very hydrophilic	-	+
CH18	42.36 ±1.50	Moderate hydrophobicity	2 ±0.32	very hydrophilic	-	+
CH19	28.70 ±1.53	Moderate hydrophobicity	1 ±0	Moderate hydrophobicity	(+)	+
CH21	25.03 ±4.10	Moderate hydrophobicity	2.5 ±0	very hydrophilic	(+)	+
CH22	3.54 ±2.29	very hydrophilic	3 ±0	very hydrophilic	-	+
CH23	2.55 ±1.05	very hydrophilic	0.5 ±0.12	Moderate hydrophobicity	+	+
CH24	14.98 ±1.54	very hydrophilic	1 ±0	Moderate hydrophobicity	(+)	+
CH25	8.77 ±0.68	very hydrophilic	3 ±0	very hydrophilic	(+)	+
CH26	8.77 ±0.68	very hydrophilic	3 ±0	very hydrophilic	(+)	+
CH27	1.58 ±0.10	very hydrophilic	2 ±0.2	very hydrophilic	-	+
CH28	8.77 ±0.68	very hydrophilic	3 ±0	very hydrophilic	(+)	+
CH29	16.01 ±2.69	very hydrophilic	2.5 ±0	very hydrophilic	+	+
CH30	12.08 ±0.09	very hydrophilic	1 ±0.32	Moderate hydrophobicity	-	+
CH33	72.25 ±6.16	very hydrophobic	2.5 ±0	very hydrophilic	-	+
CH34	12.73 ±1.48	very hydrophilic	3 ±0	very hydrophilic	(+)	+

* BATH assay described by Rosenberg *et al.*, 1980

[#] SAT assay described by (Sorongon *et al.*, 1991; and Rozgonyi *et al.*, 1985).

[†] (++) = very thick capsule; (+) = thin capsule; (-) = no capsule

BATH values for the *Chryseobacterium* and *Elizabethkingia* spp. isolates ranged from 0.77 to 72.25 %, with 70.6 % (24/34) of the isolates being classified as very hydrophilic, 26.5 % (9/34) moderately hydrophobic, and only 3.0 % (isolate CH33) as very hydrophobic (Table 3.8). The three isolates (CH10, CH23, CH24) which were considered non-adherent in nutrient-poor adherence assays were very hydrophilic (Table 3.8), while the only hydrophobic isolate (CH33) displayed positive biofilm formation under all conditions (Table 3.4 and 3.8). Isolate CH2B, the most hydrophilic isolate (0.77%), displayed positive biofilm formation under all conditions with very strong adherence in TSB, especially at 37 °C (Tables 3.4 and 3.8). No significant correlation could be observed between biofilm formation and hydrophobicity measured using the BATH assay.

3.3.2.2 *Salting Aggregation Test (SAT)*

SAT values for the *Myroides* and *Empedobacter* spp. isolates ranged from 2.5 – 3 M, and all the isolates were classified as being very hydrophilic (Table 3.8). As for the BATH assay, it was not possible to correlate biofilm formation or biofilm-forming capacity of the study isolates at room temperature/37 °C in TSB/EAOB with the SAT hydrophobicity of the isolates. Similarly, no correlation was observed between hydrophobicity of the isolates calculated by BATH and SAT assays.

Of the *Chryseobacterium* and *Elizabethkingia* spp. isolates, 19/34 (55.88 %) were classified as being very hydrophilic with SAT values ranging from 1.5 – 4 M, and 15/34 (44.12 %) displayed moderate hydrophobicity, with values of 0.2 – 1.0 M. Isolate CH2 displayed the lowest SAT hydrophobicity value (0.2 M), as well as weak adherence in the adherence assay (Tables 3.4 and 3.8). As with the BATH assay, isolate CH2B displayed the highest (4 M) hydrophilicity and displayed positive biofilm formation under all conditions with very strong adherence in TSB at 37 °C (Tables 3.4 and 3.8). Positive correlations were observed between hydrophobicity by the SAT assay and biofilm formation as well as biofilm-forming capacity, respectively, of *Chryseobacterium/Elizabethkingia* spp. isolates in nutrient-poor EAOB medium at room temperature under dynamic/static conditions ($r = 0.5192, p = 0.002$; $r = 0.5183, p = 0.002$; $r = 0.4571, p = 0.0066$; $r = 0.4653, p = 0.0056$). A similar relationship was observed for SAT

hydrophobicity and biofilm formation and biofilm-forming capacity, respectively, for isolates in nutrient-rich TSB at 37 °C under dynamic conditions ($r = 0.3646$, $p = 0.034$; $r = 0.3749$, $p = 0.029$). No significant correlation was observed between hydrophobicity determined by SAT and BATH assays.

3.4 Discussion

Myroides and *Empedobacter* spp. study isolates displayed strong adherence profiles and high relative biofilm-forming capacity, with a preference for adherence at lower temperatures (~21 °C) and nutrient-rich environments with low hydrodynamic forces (static incubation). These conditions correlated with their area of isolation, as aquacultural tanks are generally kept at lower temperatures (ambient temperature), with high nutrient availability and steady slow water flow.

Species of the genus *Myroides* have been isolated from aquatic sources (Hugo *et al.*, 2005) including South Atlantic fish spp., freshwater fish skin and in the air during chill storage of freshwater fish (González *et al.*, 2000). *Myroides* species have also been isolated from surface-associated structures or consortia including *M. pelagicus* from crude oil-utilizing and -emulsifying consortia in seawater (Maneerat *et al.*, 2005) and *M. odoratus* from mixed-species biofilm communities developed on the surfaces of seafood-processing plant equipment (Bremer *et al.*, 2001; and Tide *et al.*, 1999). In the present study, isolates also displayed moderate adherence at 37 °C in nutrient rich-environments under dynamic conditions. These conditions simulate warm-blooded animal host environments with higher hydrodynamic properties *in vitro*, including areas such as human eyes, internal organs, blood, cavity fluids and wounds from which members of these genera have been isolated. *Empedobacter* strains have been isolated from human eyes, bronchial secretions, peritoneal fluid, dialysis fluid, serous cavity fluid, cervixes and vaginas, wounds, blood and urine (Bruun, 1982; and Holmes *et al.*, 1978), while *Myroides* spp. have been isolated from human intestine, urine, faeces, wound discharge, sputum and blood (Hugo *et al.*, 2005; and Schreckenberger, 1998). *M. odoratus* has been isolated from soft tissue in amputation sites and from patients suffering from urinary tract infections (Holmes *et al.*, 1979). Similarly, Yagci *et al.* (2000) isolated *M. odoratimimus*

from urinary tract infections. The strong adherence profile displayed by study isolates was found to commence within the first 16 hours of incubation, indicating their strong affinity for adherence to surfaces as opposed to planktonic existence in nutrient-poor environments, resulting in better utilization of limited nutrients.

Although the presence of thick capsular material has previously been correlated to the strong adherence phenotype (Ofek *et al.*, 2003), it was not possible to correlate this qualitative characteristic with adherence in the present study. Quantitative characterization of the capsule might provide clues to the capsules' roles in *Myroides* and *Empedobacter* spp. adherence. All the *Myroides* and *Empedobacter* spp. isolates displayed very hydrophilic profiles by both SAT and BATH assays, but no significant differences were observed between biofilm-formation and hydrophobicity assays, as well as between the SAT and BATH assays. Thus, in this study we could not show that hydrophobicity plays a significant role in the adherence of *Myroides* and *Empedobacter* spp. isolates to polystyrene surfaces.

Outer membrane proteins may serve as adhesins anchored on the outer membrane surface of Gram-negative bacteria (Ofek *et al.*, 2003). These adhesins play an important role in virulence of bacteria as they have been found to mediate intimate adhesion to the target cell by bacteria and are often associated with invasion of the target cell or formation of a lesion at the adhesion site (Ofek *et al.*, 2003). In the previous chapter, distinction between the *Myroides* and *Empedobacter* spp. study isolates was made by the presence of an extra OMP band present in the protein profiles of two of the study isolates MY1 and MY3 (Section 2.3.7). No significant difference in biofilm formation or relative biofilm-forming capacity was observed for these isolates, in comparison to other *Myroides* spp. isolates eliminating the possibility of these proteins playing a significant role in adhesion to polystyrene surfaces. These two isolates did however show lower hydrophobicity values ($\pm 1\%$) compared to the other *Myroides* and *Empedobacter* spp. isolates, and their increased hydrophilicity may potentially be correlated to the altered OMP profiles. Further investigation into the effect of this OMP might reveal the role of the protein in the hydrophilicity of the organism.

Adherence of the *Myroides* and *Empedobacter* spp. study isolates was not influenced by the spent medium supernatant from *L. monocytogenes* or *L. innocua*.

However it has been found that the attachment of *L. monocytogenes* to stainless steel food surface areas was increased by the presence of *M. odoratus* strains (Bremer *et al.*, 2001), thus it is possible that the *Myroides* spp. isolates may produce the inducing factor when *L. monocytogenes* strains are present in their immediate environment.

Chryseobacterium and *Elizabethkingia* spp. isolates showed variation in their biofilm-forming profiles and relative biofilm-forming capacities depending on incubation temperatures and growth media used. As with the *Myroides* and *Empedobacter* study isolates, best adherence by majority of the isolates was documented at ambient temperature (~21 °C) under low hydrodynamic conditions (static incubation) in nutrient-rich environments, correlating to conditions from the aquaculture isolation sites. In a study by Basson *et al.* (in press) on the adherence abilities of *Flavobacterium* spp. isolates isolated from South African aquaculture systems, a preference for biofilm formation by the *Flavobacterium* spp. isolates was observed at 26°C, in nutrient-poor EAOB medium, under dynamic flow conditions, although they were able to form biofilm structures to a lesser extent in nutrient-rich TSB. The significance of these results is that both genera belong to the family *Flavobacteriaceae*, yet display differences in their adherence abilities. *Chryseobacterium* spp. are part of the normal microflora of aquatic environments and are often isolated from mucus on the surface of the skin, gills and intestines of fish (Bernardet *et al.*, 2006). They have been found colonizing healthy eel, skin and muscle from wild and farmed freshwater fish and intestines of salmon (*Oncorhynchus keta*) but were not implicated in causing disease in these fish during the investigations (Lijnen *et al.*, 2000; and Morita *et al.*, 1997). As opportunistic pathogens, they cause disease in fish with low immunity due to environmental stress or suffering from underlying disease caused by primary fish pathogens. In addition, they have been implicated in fish product spoilage during processing of the products as well as during storage of the fish products (Bernardet *et al.*, 2006; de Beer *et al.*, 2006; and González *et al.*, 2000). Their ability to adhere to surfaces in aquaculture cultivation tanks increases the risk of secondary infection of cultivated fish or spoilage of fish products during processing, contributing to aquacultural losses.

Chryseobacterium and *Elizabethkingia* spp. study isolates also displayed weak to moderate, and a few strong adherence profiles at 37 °C in nutrient-rich environments but

contrary to the *Myroides* and *Empedobacter* isolates, preferred lower hydrodynamic conditions which resulted in better adherence capabilities. An exception, isolate CH2B, which previously clustered with the *E. meningoseptica* reference strain through 16S rRNA PCR-RFLP analysis (Section 2.3.3), displayed the highest biofilm formation value obtained in this study at 37 °C in nutrient-rich media under dynamic conditions. This isolate may thus play a significant role in warm-blooded animal biofilm-associated disease. Species of these genera have been associated with many infections in immunocompromised and post-operative patients (Lee *et al.*, 2006; Bernardet *et al.*, 2005; Hoque *et al.*, 2001; Hsueh *et al.*, 1997; and Hsueh *et al.*, 1996). Therefore, the ability of these environmental study isolates to adhere in conditions similar to that of the human body (e.g., 37 °C and an environment rich in nutrients), may pose a threat to the health of workers handling aquaculture stock and the related fish products, especially from fish like blue tilapia. In addition, the dispersal of these isolates to veterinary or medical environments increases the threat of disease and adherence under suitable conditions could lead to difficulty in eradication of adherent or biofilm-associated *Chryseobacterium* and *Elizabethkingia* spp. organisms. In addition, adherent isolates displayed very high MAR indices and antimicrobial susceptibility test results suggested the possibility of MDR phenotypes by these isolates (section 2.3.5).

Molecules in the spent medium supernatant of other bacterial species (potentially including various quorum sensing AHLs) did not induce significant increased adherence of study isolates which were capable of initial strong adherence without induction. Weakly adherent isolates were induced to stronger adherence by growth in media supplemented with supernatant containing spent media from *Aeromonas* spp., *E. tarda*, *E. coli*, *Flavobacterium* spp. isolate YO59, *Listeria* spp., *P. aeruginosa*, *S. enterica* serovar Arizonae, and a *Vibrio* spp. isolate. Therefore, it is likely that weakly adherent *Chryseobacterium* spp. or *Elizabethkingia* spp. organisms may be incorporated into biofilms more readily in the presence of these species and contribute to an overall increased risk of medical and veterinary infection and disease, secondary infections in the aquacultural environment, contamination and spoilage of food products as well as industrial or potable water system contamination. Although the specific molecules causing induction of adherence were not identified, certain mechanisms and biological

molecules have previously been described. Wentworth *et al.* (1991) found that dying cells of *P. aeruginosa* release intracellular lectins which tether intact bacteria to each other or to the underlying surfaces through carbohydrate-lectin interactions. Members of the *Cytophaga-Flavobacterium-Bacteriodes* to which the family *Flavobacteriaceae* belong live on material released from dead microorganisms due to their ability to produce diverse enzymes (O'Sullivan *et al.*, 2002). It has also been proposed that secreted polysaccharides of the biofilm matrix may trap additional bacteria, contributing to the maturation of a biofilm community (Ofek *et al.*, 2003). Furthermore, Hasman *et al.* (1999) found that *E. coli* expresses Ag43 protein which induces the autoaggregation of nonfimbriated bacteria. *P. aeruginosa* secretes a polyuronic acid, alginate, which promotes accumulation of biofilm bacteria and biofilm development (Costerton *et al.*, 1995). Another possible mechanism that may be employed by the inducing bacteria could be similar to that of staphylococci which secrete an intracellular protein and binds to bacterial surfaces or to components of the extracellular matrix in order to enhance the adhesion of microorganisms to the target substrata (Palmer *et al.*, 2003). In *P. aeruginosa* two quorum sensing signaling systems, *lasR-lasI* and *rhlR-rhII*, are involved in biofilm formation (Davies *et al.*, 1998).

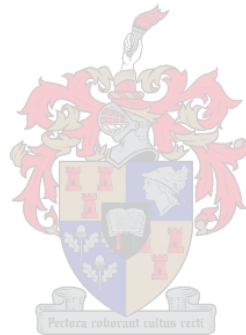
Some bacteria may undergo time-dependent changes in avidity of adhesion (Ofek *et al.*, 2003). In the present study, it was found that the majority of the *Chryseobacterium* and *Elizabethkingia* spp. study isolates adhered to the surface within the first 16 hours of incubation. By comparison, the *Vibrio* spp. control showed increased biofilm formation as duration increased. This indicates the affinity of the isolates to live in sessile state rather than having planktonic existence when nutrient availability is limited. This lifestyle mode, i.e., biofilm formation, allows nutrient access and utilization in nutrient poor environments (Ofek *et al.*, 2003).

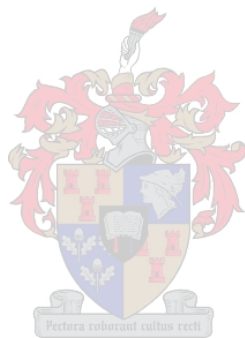
No correlation was observed between hydrophobicity as determined by the BATH assay, and the ability of the *Chryseobacterium* and *Elizabethkingia* isolates to adhere, as non-biofilm-forming and strong biofilm-forming isolates displayed very hydrophilic to moderate hydrophobicity profiles. A positive correlation with a significant difference was observed between the SAT and growth of the isolates in nutrient-poor media at room temperature under dynamic and static conditions. Similarly, a significant positive

correlation was observed between SAT and growth of the isolates in nutrient-rich media at 37 °C under dynamic conditions. Basson *et al.* (in press) found a weak negative correlation for biofilm-forming capacity of *Flavobacterium* spp. isolates and BATH hydrophilicity following growth in nutrient-poor EAOB, once again indicating the differences in adherence and cell-surface properties possibly involved in adherence and biofilm formation of the genera in the family *Flavobacteriaceae*. In addition, no correlation could be made between hydrophobicity as determined by SAT and BATH assays. This was also observed for SAT and BATH assay hydrophobicity results for *Flavobacterium* spp. isolates from similar aquaculture systems (Basson *et al.*, in press). These hydrophobicity assays often fail to correlate (Ofek *et al.*, 2003; and Babelona *et al.*, 2001), which might be explained by the SAT assay measuring the hydrophobicity of the outer surface as a whole, while the BATH assay, measured it in terms of adhesion (Mattos-Guaraldi *et al.*, 1999). Additionally, hydrophobicity and surface charge of bacteria may differ between species, serotypes or strains, change with variation in growth conditions, physiological state of cells, and composition of suspension media, or might involve variable expression of surface-associated proteins between strains (Mattos-Guaraldi *et al.*, 1999; and Sorongon *et al.*, 1991). Capsule components have been shown to play an important role in the adhesion and biofilm formation of certain bacterial species (Bell, 2001; and Decostere *et al.*, 1999). The expression of a capsule layer by 76 % of the *Chryseobacterium* / *Elizabethkingia* spp. isolates could not be correlated to the different adherence abilities or the hydrophobicity demonstrated by the study isolates. Ofek *et al.* (2003) have observed that the presence of a capsule may be responsible for masking hydrophobic surface components, thus influencing BATH test results.

The present study has revealed that the majority of the study isolates were capable of initial adherence to surfaces, which may be correlated to biofilm-formation. It was found that the presence of molecules or substances produced by human and aquaculture bacterial pathogens may induce the adherence of non-adherent or weakly adhering species of these genera. No definite correlations could be made between adherence and the hydrophobicity characteristics demonstrated by the isolates, or the genotypes/phenotypes displayed by *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates in sections 2.3.3 to 2.3.7.

Pure culture biofilms are not found in nature, and in reality consist of a diversity of different microorganisms. The composition of these mixed biofilms is a result of the autoaggregation and coaggregation characteristics demonstrated by the organisms. The role of autoaggregation and/or coaggregation was thus investigated for *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates.





CHAPTER FOUR

Biofilm maturation through aggregation of biofilm bacteria. Coaggregation and autoaggregation studies of *Chryseobacterium*, *Elizabethkingia*, *Myroides* and *Empedobacter* spp. bacteria isolated from aquaculture systems.

4.1 Introduction

After primary adherent organisms have successfully attached to a surface, they begin to divide and their progeny cover the surface of the substratum. Subsequent organisms then adhere to the primarily attached cells, with this attachment being facilitated through either coaggregation or autoaggregation (Rickard *et al.*, 2003a). Coaggregation is the adherence of genetically distinct organisms and could either be intergeneric as well as inter- and intraspecies aggregation (Rickard *et al.*, 2003b). Autoaggregation has been described as a 'selfish' ploy by which organisms aggregate with their own species so as to reach a competitive advantage within a community (Rickard *et al.*, 2003a). Both coaggregation and autoaggregation lead to the formation of microcolonies and subsequently, contribute to the maturation of a biofilm community (Rickard *et al.*, 2003b).

Coaggregation was first recognized between different oral plaque-forming bacteria, where both intergeneric and intrageneric coaggregation was observed (Kolenbrander *et al.*, 1999; and Gibbons and Nyaard, 1970). This phenomenon is now recognized in diverse environments including amongst bacteria isolated from aquatic environment biofilms (Buswell *et al.*, 1997), mammalian gut biofilms, the human urogenital tract and potable-water-supply systems (Handley *et al.*, 2001).

When considering the benefits coaggregation confers on bacterial partnerships, it is likely that the strength and specificity of the interactions will be subject to natural selection. Given that most bacteria exist in environments with fluctuating conditions (e.g., shear forces, nutrient availability or physiological conditions), bacteria within coaggregated communities will survive and proliferate under conditions that reduce the prevalence of single non-coaggregated cells (Rickard *et al.*, 2003a). Shear force can

select for coaggregation ability within a multi-species biofilm community, thus Elvers *et al.* (1998) proposed that coaggregation interactions may enhance the development of biofilms in fast-flowing water systems as non-coaggregating organisms would be washed away from their optimum ecological niche. Majority of microorganisms integrating into biofilm communities will possess physiological and biochemical characteristics that support the integration and survival of these organisms within biofilms (Handley *et al.*, 2001). The ability to coaggregate may be a significant physiological characteristic of bacteria in biofilms under high shear, as non-coaggregating bacteria are less likely to successfully integrate into developing biofilms (Handley *et al.*, 2001). Rickard *et al.* (2003b) conducted a study in a water tank with a localized shear force directed across the biofilm surface with the surrounding body of water almost static, and found coaggregating strains occurring in a much higher frequency in the freshwater multispecies biofilm than in the surrounding bulk liquid. Coaggregation is likely to enhance the development of freshwater multi-species biofilms (Rickard *et al.*, 2003b).

Another benefit is that coaggregating cells on a substratum can possess a combined metabolic advantage over single cells. Palmer *et al.* (2003) demonstrated that the coaggregating partnership of *S. oralis* and *A. naeslundii* formed a nutritionally beneficial, mutualistic relationship that allowed each to grow where neither grew alone. This mutualism is a synergistic action of several species with overlapping patterns of enzyme activity to catabolise complex organic materials and results in the liberation of additional nutrients, which might help to maintain the characteristic diversity of biofilm communities found in many habitats (Bradshaw *et al.*, 1994). Clearly in such relationships, the close proximity of the participating organisms, brought together through coaggregation, would maximize the efficiency of the consortium (Rickard *et al.*, 2003a).

Since coaggregation can take the form of intra-, inter-, or multigeneric interactions (Buswell *et al.*, 1997), a combination of these interactions contributes to the overall structure of the bacterial community in dental biofilms (Kolenbrander, 1988). Both intra- and inter-species aggregation interactions play a major role in the formation of biofilms (Shen *et al.*, 2005). Intergeneric coaggregation is common between oral bacteria (Kolenbrander and London, 1993), but intraspecies coaggregation has been

observed to occur between freshwater biofilm bacteria but has not yet been reported between plaque bacteria. Thus, intraspecies coaggregation may well be a characteristic that is unique to freshwater biofilm bacteria (Rickard *et al.*, 2002a).

Coaggregation is a highly specific process which often involves the interaction of complementary bacterial surface molecules functioning as adhesins and receptors (Kolenbrander, 1995). Examples of bacterial structures or molecules involved in coaggregation include fimbriae from numerous oral species, such as *S. sanguis*, *A. viscosus* and *P. gingivalis*, protein adhesins on the surface of *Capnocytophaga gingivalis* and *F. nucleatum*, and a carbohydrate receptor on *S. sanguis* (Kinder and Holt, 1994). Aggregation occurs through adhesin-receptor interactions, where the adhesins are lectin-like proteins and the receptors contain carbohydrates such as galactose, galactosamine or lactose residues (Kolenbrander, 2000; Rickard *et al.*, 2003b; Rickard *et al.*, 2002a; Clemans *et al.*, 1999; and Cisar *et al.*, 1979). Lectin-saccharide interactions mediating coaggregation are very common in both oral and aquatic biofilm communities (Malik *et al.*, 2003; Rickard *et al.*, 2003b; and Kolenbrander, 2000). Galactosides are the sugars most commonly recognized by lectins of oral bacteria (Kolenbrander, 2000) and aquatic bacteria (Rickard *et al.*, 2000). Coaggregation of aquatic bacteria may be reversed by the addition of simple sugars (Rickard *et al.*, 2003b; and Buswell *et al.*, 1997), which allows identification of the type of carbohydrate receptor as well as the receptor bearing cell. In addition, Kolenbrander (1995) proposed the treatment of coaggregating partners with either protease or heat to identify the coaggregating partner being the protein adhesin.

In this chapter, the coaggregation and autoaggregation abilities of members of the genera *Chryseobacterium* and *Elizabethkingia* and *Myroides* and *Empedobacter* were investigated to determine their influence in biofilm communities. Strongest coaggregating partners were subjected to reversal studies so as to potentially elucidate adhesin-receptor interactions.

4.2 Materials and Methods

4.2.1 Autoaggregation and coaggregation assays

Three *Myroides* and *Empedobacter* spp. isolates (MY1, MY2B, MY3B), and 20 *Chryseobacterium* and *Elizabethkingia* spp. isolates (CH1, CH2B, CH3, CH4B, CH8, CH11, CH12, CH15, CH18, CH21, CH22, CH23, CH25, CH26, CH28, CH29, CH30, CH33 and CH34) were examined for their ability to coaggregate, and all of the study isolates were examined for their ability to autoaggregate. The 23 selected isolates were subjected to coaggregation assays with the following bacterial partner strains, i.e., *A. hydrophila*, *A. sobria*, *A. salmonicida*, *A. media*, *S. enterica* serovar Arizonae, *Acinetobacter* spp., *E. faecalis* ATCC 2912, *E. coli*, *F. johnsoniae*-like spp. isolates YO12, YO19, YO51, YO60, YO64, *L. monocytogenes*, *L. innocua* LMG 13568, *Micrococcus luteus*, *P. aeruginosa* and *S. aureus* ATCC 25923. Study isolates and potential coaggregation partners were grown in 50 ml Erlenmeyer flasks, containing 20 ml EAOb or TSB and harvested after 36 h, by centrifugation for 10 min at 10 000 rpm. Cells were washed and resuspended in sterile distilled H₂O. Cell suspensions were standardized to an OD of 0.3 at a wavelength of 660 nm (Malik *et al.*, 2003).

In order to measure the percentage of autoaggregation, a sample of 1 ml bacterial suspension was transferred to a sterile plastic 2 ml cuvette and the OD was measured after 60 min at room temperature using a DU 640 spectrophotometer (Beckman Coulter) at a wavelength of 660 nm (Malik *et al.*, 2003). The degree of autoaggregation of each of the 39 isolates tested was determined as the percent decrease of optical density after 60 min using the equation:

$$\% \text{ Autoaggregation} = \frac{OD_o - OD_{60}}{OD_o} \times 100,$$

where OD_o refers to the initial OD of the organism, while OD₆₀ refers to the OD of the supernatant following 60 min incubation at room temperature and centrifugation at 2000 rpm for 2 min (Malik *et al.*, 2003).

The degree of coaggregation was determined by OD readings of paired isolate suspensions (500 µl of each isolate). Cell mixtures were centrifuged at 2000 rpm for 2 min and the OD of the supernatant (600 µl) was measured at a wavelength of 660 nm

(Malik *et al.*, 2003). The quantitative coaggregation rate of paired isolates was calculated using the equation:

$$\% \text{ Coaggregation} = \frac{OD_{Tot} - OD_S}{OD_{Tot}} \times 100,$$

where OD_{Tot} refers to the initial OD, taken immediately after the relevant strains were paired; and OD_S refers to the OD of the supernatant, following centrifugation of the mixture after a 60 min incubation period at room temperature (Malik *et al.*, 2003). Experiments were carried out in triplicate on two separate occasions.

4.2.2 Reversal and inhibition of coaggregation

Isolate CH2B was selected in order to investigate the effect of simple sugars, heat and protease treatment on its ability to coaggregate with *L. innocua* and *L. monocytogenes*.

4.2.2.1 Reversal with simple sugars

The ability of sugars to reverse coaggregation assays were conducted according to the method described by Rickard *et al.* (2003b). Filter-sterilized solutions of lactose and galactose, respectively, were added to coaggregating partners to final concentrations of 50 mM. Mixtures were vortexed and tested for coaggregation using the method described in section 4.2.1.

4.2.2.2 Inhibition of coaggregation by heat treatment

The ability of heat treatment to inhibit coaggregation was conducted using the method of Kolenbrander *et al.* (1985). Cells were harvested from O/N EAOB/TSB cultures, washed three times and resuspended in de-ionized water. Bacterial suspensions were then heated at 80°C for 30 min in a waterbath. Following heat treatment, the OD of each bacterial suspension was adjusted to 0.3 at a wavelength of 660 nm. The capacity of heat-treated cells to coaggregate was assessed with the method described in section 4.2.1.

4.2.2.3 *Inhibition of coaggregation by protease treatment*

Protease sensitivity of the polymers mediating coaggregation on each of the members of coaggregating pairs was tested using a method described by Rickard *et al.* (2004a). Cells were harvested from O/N EAOb/TSB cultures and resuspended in de-ionized water to an OD of 0.3 at a wavelength of 660 nm. Proteinase K was added to the standardized cell suspensions to a final concentration of 2 mg/ml. Incubation at 37°C for 2 h was followed by centrifugation and washing of the pelleted cells three times in de-ionized water. Cells were resuspended and the OD adjusted to 0.3 at 660 nm. Protease-treated and untreated cells were combined and the capacity to coaggregate determined using the method described in section 4.2.1.

4.3 Results

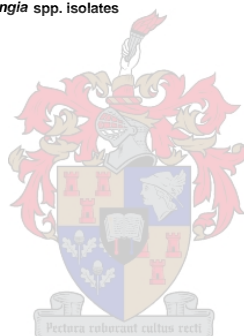
4.3.1 *Autoaggregation assays*

Autoaggregation indices of the *Myroides* and *Empedobacter* spp. isolates ranged from 7.0 – 24.8% (Table 4.1). Autoaggregation enabled the differentiation of the *Myroides* and *Empedobacter* spp. isolates into two groups based on the percentage autoaggregation where group 1 (isolates MY1, MY3) and group 2 (isolates MY2, MY2B, MY3B) displayed approximately 20-25% and 7-9% autoaggregation, respectively (Table 4.1). A significant correlation was observed between autoaggregation and biofilm formation as well as relative biofilm-forming capacity in nutrient-rich media at room temperature under dynamic conditions (biofilm formation – $r = 0.9629$, $p = 0.008$; relative biofilm-forming capacity – $r = 0.9629$, $p = 0.0085$) (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA). However, no significant correlations were observed with the BATH and SAT hydrophobicity assays. In addition the isolate clusters observed in autoaggregation correlated to the OMP profiles obtained in Chapter 2 (Table 2.9).

Autoaggregation for the *Chryseobacterium* and *Elizabethkingia* spp. isolates appeared to be strain-specific with indices ranging from 2.9 – 51.6% (Table 4.1).

Table 4. 1 Autoaggregation indices of the *Myroides* and *Empedobacter*, and *Chryseobacterium* and *Elizabethkingia* spp. isolates and 17 bacterial partner strains selected for coaggregation assays.

Bacterial isolates	Autoaggregation (%)*
<i>Acinetobacter</i> spp.	25.4
<i>Aeromonas salmonicida</i>	41.8
<i>Aeromonas hydrophila</i>	28.3
<i>Aeromonas media</i>	20.3
<i>Aeromonas sobria</i>	27.5
<i>Enterococcus faecalis</i> ATCC 9212	45.0
<i>Flavobacterium johnsoniae</i> -like isolates	
YO12	33.9
YO19	16.1
YO51	13.9
YO60	27.5
YO64	20.1
<i>Listeria innocua</i> LMG 13568	56.2
<i>Listeria monocytogenes</i>	28.9
<i>Micrococcus luteus</i>	51.1
<i>Pseudomonas aeruginosa</i>	24.3
<i>Salmonella enterica</i> serovar Arizonae	71.9
<i>Staphylococcus aureus</i> ATCC 25923	76.1
<i>Myroides</i>/<i>Empedobacter</i> spp. isolates	
MY1	22.4
MY2	7.0
MY2B	8.8
MY3	24.8
MY3B	7.7
<i>Chryseobacterium</i>/<i>Elizabethkingia</i> spp. isolates	
CH1	17.8
CH1B	47.0
CH2	41.2
CH2B	37.4
CH3	19.1
CH4	23.6
CH4B	19.9
CH5	38.9
CH6	2.9
CH7	15.4
CH8	23.8
CH9	51.6
CH10	41.3
CH11	26.3
CH12	25.9
CH13	41.2
CH14	43.5
CH15	20.4
CH16	45.7
CH17	46.2
CH18	20.4
CH19	42.7
CH21	28.8
CH22	14.0
CH23	15.7
CH24	30.1
CH25	21.3
CH26	14.6
CH27	32.0
CH28	17.9
CH29	15.1
CH30	14.9
CH33	22.6
CH34	24.9



* Autoaggregation indices represent the mean of two independent experiments carried out in triplicate at OD 660 nm (Malik *et al.*, 2003).

Strongly adherent isolates had autoaggregation indices of 21.3 – 43.5%, moderately adherent isolates 14.9 – 41.2%, weakly adherent isolates 2.9 – 47.0%, and non-adherent isolates 14.0 – 51.6%. A significant positive correlation was observed between autoaggregation and biofilm formation as well as relative biofilm-forming capacity in nutrient-rich media at room temperature under dynamic conditions (biofilm formation – $r = 0.3501$, $p = 0.0424$; relative biofilm-forming capacity – $r = 0.3505$, $p = 0.0421$) (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA). In addition, no significant relationships between autoaggregation and hydrophobicity by the BATH and SAT assays were obtained.

4.3.2 Coaggregation assays

Coaggregation indices of the *Myroides* and *Empedobacter* spp. isolates with all 17 of the selected strains were relatively low and ranged from 6.8 – 42.4% (Table 4.2). The highest coaggregating partner was *S. aureus* ATCC 25923 with coaggregation indices ranging from 30.8 – 42.4% (Table 4.2). Their second highest partner was *Enterococcus faecalis* ATCC 9212 with coaggregation indices ranging from 30.9 – 35.0% (Table 4.2). No significant differences or correlations (ANOVA; Statistica v.7.0, Statsoft, Tulsa, USA) were obtained between coaggregation assay results and the phenotypic or genotypic typing technique results obtained in Chapter 2 (Table 2.9). In addition, no significant correlations could be made between the autoaggregation and coaggregation indices of the study isolates (ANOVA, t-tests).

Coaggregation occurred to varying degrees with all of the 17 partner strains, and the 20 selected *Chryseobacterium* and *Elizabethkingia* spp. isolates (Table 4.3). Coaggregation indices ranged from 1.10 – 82.2%, with strongest coaggregation displayed with *E. faecalis* ATCC 9212, *S. enterica* serovar Arizonae, *L. monocytogenes*, *L. innocua* LMG 13568, and *S. aureus* ATCC 25923. Both strong- (isolates CH25, and CH34) and weak- (isolates CH4, CH4B, CH8, CH15, CH18, CH21, CH26, and CH33) biofilm-forming isolates were able to coaggregate strongly with a variety of partner strains (Table 4.3). Therefore, it was not possible to identify isolates capable of forming moderate- to strong- biofilms structures as being more effective in coaggregation than the non-biofilm-

forming isolates. No significant differences or correlations were obtained between coaggregation assay results, autoaggregation assays, and the phenotypic or genotypic typing technique results obtained in Chapter 2 (Table 2.9).

Table 4.2 Coaggregation indices of the selected biofilm-forming and non-biofilm forming *Myroides* and *Empedobacter* spp. isolates and 17 bacterial partner strains.

<i>Myroides/Empedobacter</i> spp. isolates	Coaggregation indices (%) [*]			
	MY1	MY2B	MY3B	
Biofilm Phenotype [‡]	+++	+++	+++	
Autoaggregation Index (%) [#]	22.4	8.8	7.7	
Partner strains	Range (%)			
<i>Acinetobacter</i> spp.	6.8 - 10.5	8.6	10.5	6.8
<i>Aeromonas salmonicida</i>	13.2 - 20.7	20.7	13.2	13.2
<i>Aeromonas hydrophila</i>	8.2 - 15.0	15.0	10.0	8.2
<i>Aeromonas media</i>	9.0 - 10.1	9.0	9.4	10.1
<i>Aeromonas sobria</i>	7.8 - 13.4	13.4	11.0	7.8
<i>Enterococcus faecalis</i> ATCC 9212	30.9 - 35.0	30.9	35.0	32.1
<i>Flavobacterium johnsoniae</i> -like isolates				
YO12	12.7 - 13.8	13.8	13.1	12.7
YO19	9.1 - 9.6	9.6	9.1	9.1
YO51	8.0 - 11.4	11.4	8.0	9.3
YO60	8.4 - 9.9	9.9	8.4	9.9
YO64	8.9 - 10.5	10.5	8.9	8.9
<i>Listeria innocua</i> LMG 13568	16.4 - 18.4	18.4	16.4	17.4
<i>Listeria monocytogenes</i>	7.4 - 10.1	10.1	9.8	7.4
<i>Micrococcus luteus</i>	13.5 - 14.4	14.4	13.5	13.7
<i>Pseudomonas aeruginosa</i>	11.7 - 14.6	14.6	12.1	11.7
<i>Salmonella enterica</i> serovar Arizonae	11.4 - 12.5	12.5	11.4	12.4
<i>Staphylococcus aureus</i> ATCC 25923	30.8 - 42.4	30.8	42.4	34.7

* Coaggregation indices represent the means of two independent replicate experiments as described by Malik *et al.* (2003).

[‡] Biofilm phenotypes were determined by microtitre assays (Stepanovic *et al.*, 2000 and Van Houdt *et al.*, 2004). -, +, ++, +++ refer to no biofilm, weak, moderate and strong biofilm formation.

[#] Autoaggregation indices were determined according to assay of Malik *et al.* (2003).

Isolate CH2B was identified as being the strongest coaggregating isolate with 53% of the partner strains (Table 4.4). Isolate CH18 displayed the lowest coaggregation indices with 29% of the partner strains, and was thus designated the weakest overall partner in the coaggregation assays (Table 4.4). Isolate MY1 coaggregated strongly with approximately 70% (12/17) of the partner strains (Table 4.4).

Table 4.3 Coaggregation indices of the selected biofilm-forming and non-biofilm forming *Chryseobacterium* and *Elizabethkingia* spp. isolates, and 17 bacterial partner strains selected for coaggregation assays.

<i>Chryseobacterium/Elizabethkingia</i> spp. isolates	Coaggregation indices (%)*																				
	CH1	CH2B	CH3	CH4	CH4B	CH8	CH11	CH12	CH15	CH18	CH21	CH22	CH23	CH25	CH26	CH28	CH29	CH30	CH33	CH34	
Biofilm Phenotype [‡]	-	++	++	+	+	+	++	++	+	+	+	-	-	+++	+	++	++	++	+	+++	
Autoaggregation Index (%) [#]	17.8	37.4	19.1	23.6	19.9	23.8	26.3	25.9	20.4	20.4	28.8	14.0	15.7	21.3	14.6	17.9	15.1	14.9	22.6	24.9	
Partner strains	Range (%)																				
<i>Acinetobacter</i> spp.	7.2 - 32.7	13.06	32.71	14.54	14.37	15.27	14.86	16.29	16.46	9.60	7.21	13.61	7.41	16.23	16.12	11.85	11.47	12.29	11.92	13.16	14.52
<i>Aeromonas salmonicida</i>	12.4 - 31.6	24.50	31.58	23.07	24.17	18.02	24.66	25.65	27.00	18.80	12.38	24.10	23.29	27.38	25.84	21.22	23.93	23.42	20.85	25.18	21.20
<i>Aeromonas hydrophila</i>	10.0 - 32.4	13.03	18.06	16.86	15.40	17.17	14.80	19.28	12.77	9.99	13.20	18.35	17.90	13.24	17.24	32.43	16.54	16.17	15.94	15.16	20.84
<i>Aeromonas media</i>	12.8 - 38.0	17.64	38.03	16.87	16.07	14.73	16.90	18.93	17.70	23.22	18.77	19.76	15.83	16.04	26.06	15.60	16.06	14.57	12.84	20.10	17.96
<i>Aeromonas sobria</i>	11.0 - 27.5	11.09	27.49	14.26	11.88	14.17	19.15	15.39	17.35	13.85	15.10	10.96	13.76	14.17	16.19	14.94	13.46	14.25	17.91	13.44	21.96
<i>Enterococcus faecalis</i> ATCC 9212	32.2 - 55.7	40.58	44.32	42.71	43.16	46.53	43.70	43.70	54.08	55.68	50.66	48.89	55.73	44.21	33.26	34.28	35.56	32.21	43.22	37.85	37.28
<i>Flavobacterium</i> <i>johnsoniae</i> -like isolates																					
YO12	15.5 - 38.4	20.48	36.56	26.07	38.35	37.65	25.30	22.86	28.68	24.03	24.07	29.50	18.26	15.52	20.52	17.34	19.64	37.77	33.67	20.80	29.04
YO19	7.1 - 40.5	16.83	28.92	21.05	28.62	37.54	19.79	21.94	21.18	19.14	18.30	39.46	18.72	7.13	19.62	26.10	22.02	34.79	40.54	25.63	28.73
YO51	1.1 - 40.2	14.12	25.24	22.11	24.58	23.64	16.64	15.79	15.93	13.54	12.41	40.19	9.73	1.10	18.37	21.45	23.25	21.03	39.65	15.41	24.61
YO60	12.5 - 32.1	12.50	17.98	19.94	17.03	18.12	15.20	18.31	18.31	18.46	14.03	17.02	16.32	18.03	17.73	32.08	15.77	15.56	16.12	15.24	19.79
YO64	11.6 - 36.6	11.96	16.70	14.48	15.36	17.55	14.36	19.90	13.50	13.23	11.62	16.92	27.05	14.21	22.94	36.63	17.47	16.34	16.37	16.13	17.88
<i>Listeria innocua</i> LMG 13568	13.8 - 77.3	23.61	77.35	21.92	23.22	24.81	18.75	14.96	17.61	14.77	13.82	19.04	20.24	24.07	24.64	21.50	20.23	21.52	22.77	25.02	27.60
<i>Listeria monocytogenes</i>	12.5 - 70.4	13.62	70.36	12.61	16.09	15.21	29.77	24.85	22.39	22.15	21.23	15.15	16.74	17.03	19.02	15.13	12.51	16.30	14.61	18.90	21.05
<i>Micrococcus luteus</i>	15.4 - 37.2	21.59	37.18	21.31	22.89	20.16	23.54	23.84	24.89	22.22	15.38	22.69	24.84	24.28	23.51	18.63	18.08	20.14	20.59	19.88	17.06
<i>Pseudomonas aeruginosa</i>	12.2 - 44.1	13.66	44.13	18.61	21.67	20.83	20.09	25.34	18.11	15.85	17.05	18.11	19.72	14.07	16.98	21.53	17.91	12.20	13.54	26.62	14.26
<i>Salmonella enterica</i> serovar Arizonae	12.4 - 56.6	19.86	45.98	20.85	21.08	19.20	21.98	22.79	56.57	12.38	18.46	21.36	22.10	19.91	23.34	21.57	22.63	17.56	16.18	27.18	18.22
<i>Staphylococcus aureus</i> ATCC 25923	31.3 - 82.2	42.20	82.16	41.00	45.37	44.44	41.57	41.57	47.20	48.25	43.96	41.14	49.06	32.56	44.10	43.21	45.29	31.25	45.11	48.38	53.23

* Coaggregation indices represent the means of two independent replicate experiments as described by Malik *et al.* (2003).

[‡] Biofilm phenotypes were determined by microtitre assays (Stepanovic *et al.*, 2000 and Van Houdt *et al.*, 2004). -, +, ++, +++ refer to no biofilm, weak, moderate and strong biofilm formation.

[#] Autoaggregation indices were determined according to assay of Malik *et al.* (2003).

Table 4. 4 Study isolates displaying strongest and weakest coaggregation with 17 bacterial partner strains in coaggregation assays.

Coaggregation Partners	<i>Myroides/Empedobacter</i> spp. isolates			<i>Chryseobacterium/Elizabethkingia</i> spp. isolates		
	Range (%)	Weakest Partner	Strongest Partner	Range (%)	Weakest Partner	Strongest Partner
<i>Acinetobacter</i> spp.	6.8 - 10.5	MY3B	MY2B	7.2 - 32.7	CH18	CH2B
<i>Aeromonas salmonicida</i>	13.2 - 20.7	MY2B	MY1	12.4 - 31.6	CH18	CH2B
<i>Aeromonas hydrophila</i>	8.2 - 15.0	MY3B	MY1	10.0 - 32.4	CH15	CH2B
<i>Aeromonas media</i>	9.0 - 10.1	MY1	MY3B	12.8 - 38.0	CH30	CH2B
<i>Aeromonas sobria</i>	7.8 - 13.4	MY3B	MY1	11.0 - 27.5	CH21	CH2B
<i>Enterococcus faecalis</i> ATCC 9212	30.9 - 35.0	MY1	MY2B	32.2 - 55.7	CH29	CH15
<i>Flavobacterium johnsoniae</i> -like isolates						
YO12	12.7 - 13.8	MY3B	MY1	15.5 - 38.4	CH23	CH4
YO19	9.1 - 9.6	MY3B	MY1	7.1 - 40.5	CH23	CH30
YO51	8.0 - 11.4	MY2B	MY1	1.1 - 40.2	CH23	CH21
YO60	8.4 - 9.9	MY2B	MY1	12.5 - 32.1	CH1	CH26
YO64	8.9 - 10.5	MY2B	MY1	11.6 - 36.6	CH18	CH26
<i>Listeria innocua</i> LMG 13568	16.4 - 18.4	MY2B	MY1	13.8 - 77.3	CH18	CH2B
<i>Listeria monocytogenes</i> LMG	7.4 - 10.1	MY3B	MY1	12.5 - 70.4	CH28	CH2B
<i>Micrococcus luteus</i>	13.5 - 14.4	MY2B	MY1	15.4 - 37.2	CH18	CH2B
<i>Pseudomonas aeruginosa</i>	11.7 - 14.6	MY3B	MY1	12.2 - 44.1	CH29	CH2B
<i>Salmonella enterica</i> serovar Arizonae	11.4 - 12.5	MY2B	MY1	12.4 - 56.6	CH15	CH12
<i>Staphylococcus aureus</i> ATCC 25923	30.8 - 42.4	MY1	MY2B	31.3 - 82.2	CH29	CH2B

4.3.3 Reversal and inhibition of coaggregation

High coaggregation indices were obtained between the *Chryseobacterium* and *Elizabethkingia* spp. isolate CH2B and the *Listeria* spp. coaggregation partners (Table 4.3). Therefore, reversal and inhibition of the coaggregation partnerships were investigated with the sugars, lactose and galactose, and inhibition through heat and proteinase K treatment (Table 4.5). Sugar reversal experiments led to an overall increase in coaggregation with the *Listeria* spp. partner strains and autoaggregation of isolate CH2B (Table 4.5). Heat treatment of CH2B resulted in a decrease in autoaggregation (37.4 to 20.9%) and coaggregation (*L. innocua* – 77.3 to 33.5%; *L. monocytogenes* – 70.4 to 12.9%). However, no such a decrease was observed when the *Listeria* spp. partner strains were treated with heat (Table 4.5). Similarly, proteinase K treatment of isolate CH2B resulted in a decrease of autoaggregation by this isolate (37.4 – 25.8%) as well as coaggregation with the partner strains (*L. innocua* – 77.3 to 38.2%; *L. monocytogenes* – 70.4 to 5.8%). Proteinase K treatment of the partner strains did not result in a decrease in coaggregation (Table 4.5).

Table 4. 5 Sugar reversal, and heat and proteinase K inhibition of coaggregation between *Chryseobacterium* and *Elizabethkingia* spp. study isolate CH2B and coaggregation partners *L. innocua* and *L. monocytogenes*.

Treatment	Coaggregation (%)* Untreated Partners		
	CH2B	<i>L. innocua</i>	<i>L. monocytogenes</i>
No Treatment			
CH2B	37.4	77.3	70.4
Lactose reversal			
CH2B	68.7	96.0	89.6
<i>L. innocua</i>	94.5	-	-
<i>L. monocytogenes</i>	87.8	-	-
Galactose reversal			
CH2B	56.4	86.2	95.7
<i>L. innocua</i>	85.0	-	-
<i>L. monocytogenes</i>	95.8	-	-
Heat inhibition			
CH2B	20.9	33.5	12.9
<i>L. innocua</i>	93.8	-	-
<i>L. monocytogenes</i>	97.7	-	-
Proteinase K inhibition			
CH2B	25.8	38.2	5.8
<i>L. innocua</i>	80.4	-	-
<i>L. monocytogenes</i>	94.6	-	-

* Coaggregation indices represent the means of two independent replicate experiments as described by Malik *et al.* (2003).

4.4 Discussion

Coaggregation is a process by which genetically distinct bacteria become attached to one another through specific molecules (Rickard *et al.*, 2003a). Similarly, autoaggregation describes the aggregation of bacteria, although this attachment is between genetically identical strains (Rickard *et al.*, 2003a). Both coaggregation and autoaggregation interactions may influence the development of complex multi-species biofilms (Rickard *et al.*, 2003a).

Myroides and *Empedobacter* spp. study isolates displayed variation in autoaggregation indices which clustered the isolates into two groups. The group

displaying an approximately 10% higher autoaggregation index contained two isolates which were previously (section 2.3.7) clustered into a separate group due to the presence of an additional OMP. OMPs may serve as adhesins anchored on the outer membrane surface of Gram-negative bacteria, and have been found to mediate intimate adhesion to surfaces by bacteria (Ofek *et al.*, 2003). Since these proteins were found not to play a role in adhesion to PVC (Chapter 3), it is possible that these proteins may play a role in the recognition or adherence of genetically identical bacterial cells.

Similar to the *Myroides* and *Empedobacter* spp. study isolates, diverse autoaggregation indices were also obtained between the *Chryseobacterium* and *Elizabethkingia* spp. isolates. Autoaggregation interactions have been shown to be enhanced by increased hydrophobicity (Rickard *et al.*, 2004b). For *F. johnsoniae* a positive correlation was observed between surface hydrophobicity by the BATH assay and autoaggregation (Basson *et al.*, in press). However, no correlation between hydrophobicity and autoaggregation was observed in the present study. A positive correlation was found between autoaggregation and adherence of the *Myroides* and *Empedobacter* spp. isolates in nutrient-rich media at room temperature under dynamic conditions. For the *F. johnsoniae* study isolates, the ability to autoaggregate did not correlate with a specific biofilm formation phenotype (Basson *et al.*, in press). These comparisons indicate the diversity in adherence and biofilm formation mechanisms in the family *Flavobacteriaceae*.

Myroides and *Empedobacter* spp. isolates displayed high coaggregation indices with *E. faecalis* and *S. aureus*, bacteria important from a food microbiology and public health perspective. It has been found that the attachment of *L. monocytogenes* to stainless steel food surface areas was largely increased by the presence of *M. odoratus* strains (Bremer *et al.*, 2001). In the present study, coaggregation between the *Myroides* and *Empedobacter* spp. study isolates and *L. monocytogenes* was very low. Isolate MY1 displaying higher autoaggregation indices, was also a strong coaggregating partner with 70% of the bacterial partners. This indicates that the additional OMP displayed (section 2.3.7) by this isolate might potentially be involved in the recognition or aggregation with not only genetically identical, but also genetically distinct bacteria.

Diverse coaggregation indices were obtained for the *Chryseobacterium* and

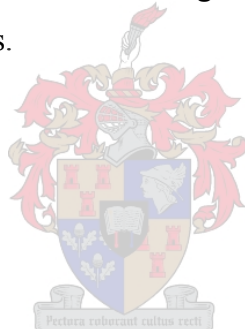
Elizabethkingia spp. isolates and respective partner strains. Unlike the *Myroides* and *Empedobacter* spp. isolates, the OMP profiles described previously (section 2.3.7) for the *Chryseobacterium* and *Elizabethkingia* spp. could not be correlated to specific autoaggregating or coaggregating profiles. Strongest coaggregation was displayed with *E. faecalis* ATCC 9212, *S. enterica* serovar Arizonae, *L. monocytogenes*, *L. innocua* LMG 13568, and *S. aureus* ATCC 25923. Similarly, in a previous study (Basson *et al.*, in press), *F. johnsoniae* isolates showed overall high rates of coaggregation against *S. enterica* serovar Arizonae, *S. aureus* ATCC 25923, *E. faecalis* ATCC 2912, and to a lesser extent to *L. monocytogenes*, *L. innocua* and *Micrococcus* spp. Therefore, it is possible that species of genera belonging to the family *Flavobacteriaceae* display specificity in their coaggregation affinity with specific strains belonging to other genera.

Biofilm induction experiments (section 3.3.1.1) revealed increased adherence of isolates CH15 and CH25 in cell-free supernatants of *A. hydrophila* and *P. aeruginosa*, and isolate CH8 in the presence of cell-free supernatants of *A. salmonicida*, *L. monocytogenes* and *P. aeruginosa*. However, intermediate coaggregation indices were observed between these isolates and the respective inducing partners, indicating that induction of increased adherence of the study isolates was due to molecules (such as those implicated in quorum sensing) released by the partner strains into the growth media, rather than molecules present on their cell-surfaces.

Study isolate CH2B coaggregated strongest with 53% of the partner strains, and displayed very high coaggregation indices with the *Listeria* spp. partner strains. Coaggregation between aquatic biofilm-forming bacteria was found to be reversed by simple sugars such as galactose, galactosamine and lactose (Rickard *et al.*, 2003a; and Buswell *et al.*, 1997). For oral bacteria, coaggregation interactions have been described as either lactose-inhibitable or lactose-non-inhibitable depending on the reversal ability of this sugar (Kolenbrander, 1995). In the present study, the addition of lactose and galactose did not reverse coaggregation, and treatment with a more diverse range of sugars is necessary in order to identify the specific carbohydrate receptor. Subsequent inhibition studies, with heat and proteinase treatment revealed isolate CH2B as the lectin bearing partner.

M. luteus, *B. natatoria*, *Fusobacterium* and *Prevotella* spp. were identified as bridging organisms in biofilms due to their ability to coaggregate with all the coaggregating partners (Rickard *et al.*, 2002a; Buswell *et al.*, 2001; Kolenbrander, 1989; and Kolenbrander *et al.*, 1985). In the present study, study isolate CH2B displayed high coaggregation indices with 10 of the 17 partner strains, and it is, therefore, not unlikely that it is a possible bridging organism in aquaculture environments.

In the present study, it was determined that *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. study isolates possessed the ability to associate with bacteria that are important from a food microbiology and public health perspective. Given their MDR phenotypes (section 2.3.5) and their ability to adhere to surfaces (Chapter 3), this is a great concern from a public health standpoint. The investigation of biofilm formation and architecture by the flow-cell studies was conducted to further elucidate the biofilm-forming capacity and interaction of the study isolates with other bacterial species.





CHAPTER FIVE

Biofilm flow cell studies

5.1 Introduction

In natural systems, such as the aquaculture system, biofilms are often located in places where access is limited, which makes direct analysis of these communities and the individual microorganisms difficult. However, just as laboratory-based batch culture experiments have been used to study the physiological behaviour of suspended bacteria, it is possible to obtain significant information about the bacterial behaviour of organisms growing in a complex biofilm using experimental biofilm model systems (Christensen *et al.*, 1999).

Microorganisms isolated from a diversity of biofilm environments (medical, environmental and industrial) have been subjected to adherence and biofilm studies (Bomo *et al.*, 2003; Mridula *et al.*, 2003; Coquet *et al.*, 2002a; Croxatto *et al.*, 2002; Wang *et al.*, 2002; and Karunasagar *et al.*, 1996). The use of several techniques have allowed for the successful recreation of biofilm communities and the study thereof. These techniques include solid supports suspended in beakers (Critchley *et al.*, 2003; Coquet *et al.*, 2002a; Wang *et al.*, 2002; Elvers *et al.*, 1998; and Karunasagar *et al.*, 1996), flow cell studies (Wang *et al.*, 2003; and Hall-Stoodley and Lappin-Scott, 1998), bioreactor studies (Banning *et al.*, 2003; and Bremer *et al.*, 2002) and molecular techniques (Oppong *et al.*, 2003; and O'Sullivan *et al.*, 2002).

Molecular techniques allow for the study of microbial communities and the genes involved in biofilm formation. O'Sullivan *et al.* (2002) examined bacterial diversity of biofilms formed on stones in River Taff and identified members of the *Cytophaga-Flavobacterium-Bacteroides* phylum in these river biofilm communities (O'Sullivan *et al.*, 2002).

The ability of microorganisms to adhere to surfaces present in medical, food, aquacultural and industrial environments has been successfully studied through suspending various materials as solid supports in beakers and allowing for biofilm

formation (Critchley *et al.*, 2003; Coquet *et al.*, 2002a; Wang *et al.*, 2002; Elvers *et al.*, 1998; and Karunasagar *et al.*, 1996). Adherence to glass and wood by fish pathogens *V. anguillarum* and *Y. ruckeri*, respectively, was demonstrated using this technique (Wang *et al.*, 2002; and Coquet *et al.*, 2002a). Similarly, the ability of the shrimp pathogen *V. harveyi* to adhere to various materials used in the aquaculture industry including concrete, stainless steel and plastic was determined by suspending these surfaces in inoculated beakers (Karunasagar *et al.*, 1996).

Bioreactors and flow systems have been devised to gain more accurate models of the dynamics of biofilms in conditions simulating that of the natural environment (Banning *et al.*, 2003; Wang *et al.*, 2003; Bremer *et al.*, 2002; and Hall-Stoodley and Lappin-Scott, 1998). Flow systems, including the Robbins' device and flow cells, generally consist of inoculated chambers or channels, which contain either removable components or are covered with glass slides for microscopic investigation. Continuous flow of fresh medium through these systems is facilitated by the use of a pump.

The Robbins' device provides quantifiable samples of biofilms growing on submerged surfaces in aqueous systems. This device has been used in studies of biofilm formation by *P. aeruginosa* and *S. aureus* isolated from patients suffering from cystic fibrosis and osteomyelitis, respectively, and in industrial studies for biofilm sampling in very high pressure oil transmission pipelines (Kharazmi *et al.*, 1999). The Robbins' device consists of removable silicone disks, although a number of other materials can be inserted (McLean *et al.*, 1999), on which biofilms can form and then be examined *in vitro* (Kharazmi *et al.*, 1999). This device is limited with regards to delineating the structural organization within the biofilm communities, since substratum needs to be removed for investigation, and often leads to destruction of spatial cellular arrangements (architecture) within the community being analyzed (Karthikeyan *et al.*, 2000). Flow cell systems allow for the direct microscopic investigation of both structural and spatial organization within intact, fully-hydrated biofilm communities, under continuous flow conditions (Karthikeyan *et al.*, 2000). Different flow cell models have been used for the study of biofilm formation, including the study of the effect of a *V. anguillarum* OMP on their biofilm formation ability (Wang *et al.*, 2003) and the study of biofilm growth by *Mycobacterium* spp. in flow cell systems where the batch culture was re-circulated at a

constant flow rate (Hall-Stoodley and Lappin-Scott, 1998). Investigations of biofilm formation in flow cell systems have been enhanced by fluorescence *in situ* hybridization and fluorescent antibody techniques in conjunction with epifluorescence or scanning confocal laser microscopy (Karthikeyan *et al*, 2000). 16S rRNA *in situ* hybridization analysis has been described for the study of mixed staphylococcal biofilms consisting of *S. aureus* and *S. epidermidis* (Ziebuhr *et al.*, 1999). Another technique includes the insertion of various Green Fluorescent Protein (GFP)-variant genes into specific biofilm members, which permits the direct analysis of the abundance and distribution of these organisms within multispecies systems over time (Karthikeyan *et al*, 2000).

In the present study, members of the genera *Myroides* and *Empedobacter* and *Chryseobacterium* and *Elizabethkingia* isolated from various aquaculture sources, displayed heterogeneity with respect to biochemical, phenotypic and genotypic phenotypes, and were able to adhere to PVC surfaces, as well as autoaggregate and coaggregate with bacterial strains important from a food microbiology and public health perspective. The following study investigated the biofilm structures in both single and mixed biofilm communities by these isolates in flow cell systems simulating their natural environment in order to investigate biofilm architecture and obtain information on their behavior and interaction with other bacterial species.



5.2 Materials and Methods

Based on the microtitre adherence assay studies, one *Myroides* and *Empedobacter* spp. study isolate (MY1) and seven *Chryseobacterium* and *Elizabethkingia* spp. study isolates (CH2B, CH8, CH15, CH23, CH26, CH25, and CH34) were selected for pure culture flow cell studies (Table 5.1). A *L. monocytogenes* strain was used together with MY1 and CH2B in the mixed-species biofilm flow cell studies.

An eight chamber continuous-flow flow cell (Wolfaardt *et al.*, 1994) constructed of Perspex (Fig. 5.1), with flow chambers 2.2 mm deep, 4 mm wide, and 31 mm long was used. A no. 1 microscope coverslip (Lasec, RSA) was attached to the Perspex with silicone adhesive. Effluent silicone tubes with an inner diameter of 1 mm and either 10 or 150 mm in length (The Silicone Tube, RSA) were attached to the flow chambers with

silicone adhesive. Plastic adapters (T-connectors, 1/16"; straight connectors, 1/16" x 1/16"; Cole-Parmer Instrument Co., USA) were used for the attachment of the silicone tubing to a reservoir containing 2 l of growth media (EAOB/TSB). Flow chambers and silicone tubing was sterilized with liquid bleach O/N and rinsed with sterile growth medium for 1 h prior to inoculation. A flow rate of 14.5 ml.h⁻¹ was maintained with a Watson Marlow 205S multichannel peristaltic pump. Sterile syringes were used to inoculate 1 ml pure O/N culture of the respective study isolates, into each chamber. In the case of mixed-species studies, 0.5 ml of the respective pure O/N cultures of the study bacterial species per chamber was inoculated into the flow cell chambers. Stagnant conditions were maintained for the first hour, before inoculated chambers were exposed to flowing growth medium, either nutrient-rich (TSB) or nutrient-poor (EAOB) media, respectively. Flow cell systems were maintained at room temperature (23 ± 2°C). Each flow cell chamber was investigated microscopically using a Nikon Eclipse E400 microscope (Nikon, Japan) after 24 h and 48 h of incubation, respectively, to visualize bacterial attachment and biofilm-formation.

Table 5.1 Selection of *Myroides* and *Empedobacter* spp. isolate and *Chryseobacterium* and *Elizabethkingia* spp. isolates for flow cell experiments based on their adherence abilities in TSB and EAOB media as determined in microtitre plate adherence assay results.

Study isolates	Microtitre Adherence Profiles* at room temperature (23 ± 2°C) under static/dynamic conditions			
	EAOB		TSB	
	Static	Dynamic	Static	Dynamic
MY1	S [‡]	S	S	S
CH2B	M [#]	M	S	S
CH8	W [¥]	W	W	M
CH15	W	W	M	M
CH23	N [§]	N	W	N
CH25	S	S	W	W
CH26	M	W	M	W
CH34	S	S	S	S

* Biofilm formation assayed according to Stepanovic *et al.* (2000).

[‡] Strongly adherent

[#] Moderately adherent

[¥] Weakly adherent

[§] Non-adherent

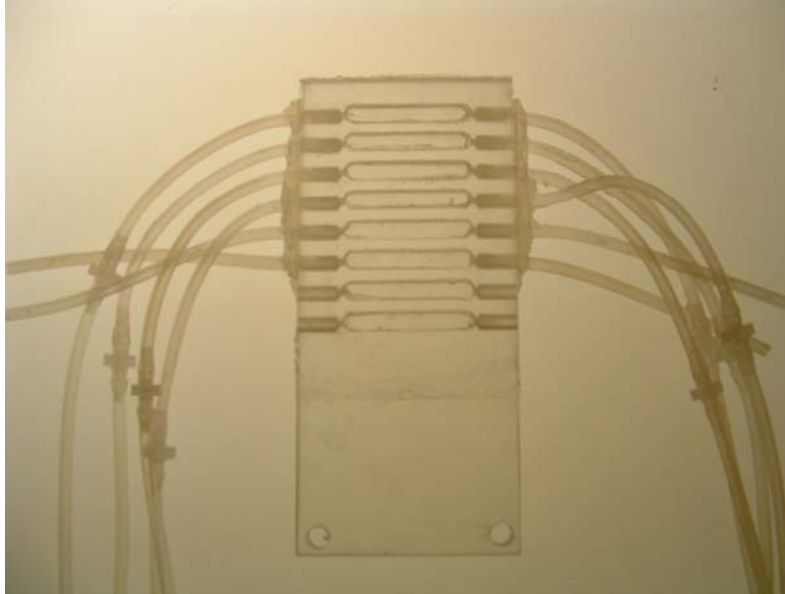


Figure 5.1 Eight channel perspex flow cell used to cultivate biofilms for microscopic examination. Flow cells are covered with a glass coverslip, connected to upstream growth medium reservoir with silicone tubes, and effluent silicone tubes to carry effluent to waste container.

5.3 Results

5.3.1 Pure culture, single species flow cell experiments

Adherence of study isolates to the glass coverslips was investigated by light microscopy, starting from the surface of the glass slide and scanning several planes interspersed by short distances in order to visualize biofilm architecture and microbial behavior throughout the depth of the individual flow chambers. Adherence to glass coverslips in nutrient-poor (EAOB) media was observed for all the study isolates within the first 24 h of inoculation. However, the ability to form biofilm structures varied between the isolates.

Myroides and *Empedobacter* spp. isolate MY1 displayed microcolony formation in the form of cone-like structures narrowing to single cell chains toward flowing media or during increased hydrodynamic conditions, within the first 24 h of incubation (Fig. 5.2). Cream-colored carpets covering the surface of the slide were observed without the microscope after 48 h incubation. Thick biofilm growth with polar attached cells (Fig. 5.3) and cone-structures reaching deep into the flowing media were observed (Fig. 5.4). In addition, individual microcolonies formed connecting structures/bridges between each

other (Fig. 5.6). Biofilm growth was thick in the middle of the glass slides, but monolayers were observed at the side where silicone adhesive came into contact with the glass surface indicating preference to hydrophilic surfaces.

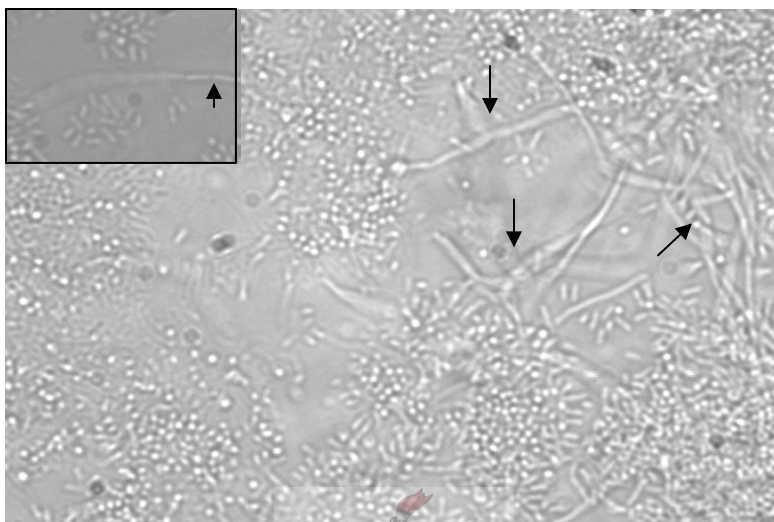


Figure 5. 2 Light microscope image depicting cone-like biofilm structures of MY1 associated with the glass slide surface, and cell chains reaching into the media (arrows) following 24 h of flow cell incubation in nutrient-poor (EAOB) media ($\times 1000$ magnification). Insertion: Image depicting cell-to-cell aggregation (arrow) to form cell chains.



Figure 5. 3 Light microscope image depicting polar attachment (spots) on the glass slide surface by study isolate MY1 following 48 h of flow cell incubation nutrient-poor (EAOB) media ($\times 1000$ magnification).

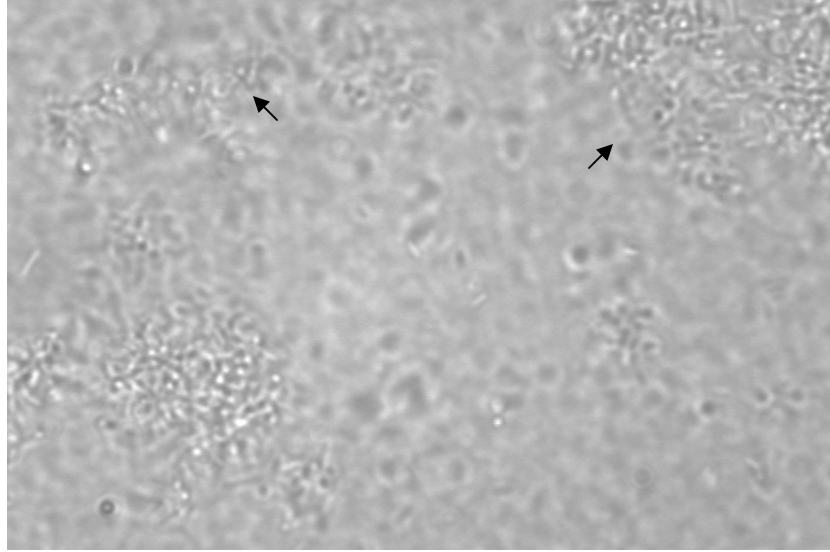


Figure 5. 4 Light microscope image depicting cone formation (arrows) of biofilm structures formed by isolate MY1 on the glass slide surface following 48 h of flow cell incubation nutrient-poor media (EAOB) ($\times 1000$ magnification).

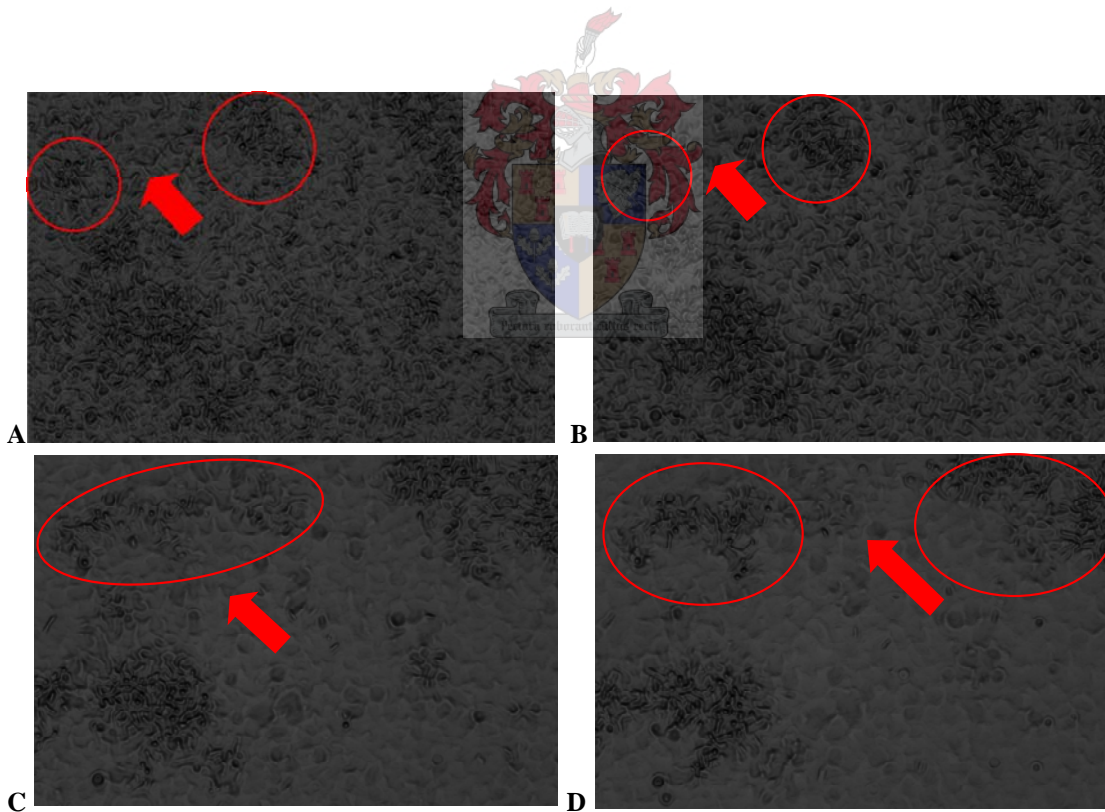


Figure 5. 5 Enhanced light microscope images (Paint Shop Pro v.7) depicting the sequence of bridge formation (A to E indicated with arrows) between microcolonies of isolate MY1 into flowing media following 48 h of flow cell incubation in nutrient-poor media (EAOB). Images were obtained by scanning several planes interspersed by short distances from the surface of the glass slide (A) into the depth of the flow chamber (D) ($\times 1000$ magnification).



E

Figure 5. 6

Enhanced light microscope images (Paint Shop Pro v.7) depicting the bridge formation between microcolonies of isolate MY1 into flowing media following 48 h of flow cell incubation in nutrient-poor media (EAOB). Images were obtained by scanning several planes interspersed by short distances from the surface of the glass slide (Fig 5.5A-D) into the depth of the flow chamber ($\times 1000$ magnification).

Isolate CH2B displayed initial widespread attachment to glass surfaces after 24 h of incubation in nutrient-poor (EAOB) media. Microcolonies with chains of cells into the hydrodynamic area were also observed (Fig 5.7). After 48 h of incubation, majority of the cells were attached at a polar end, and the typical mushroom/or cone-structures were observed with chains of cells reaching into the media (Fig 5.8).

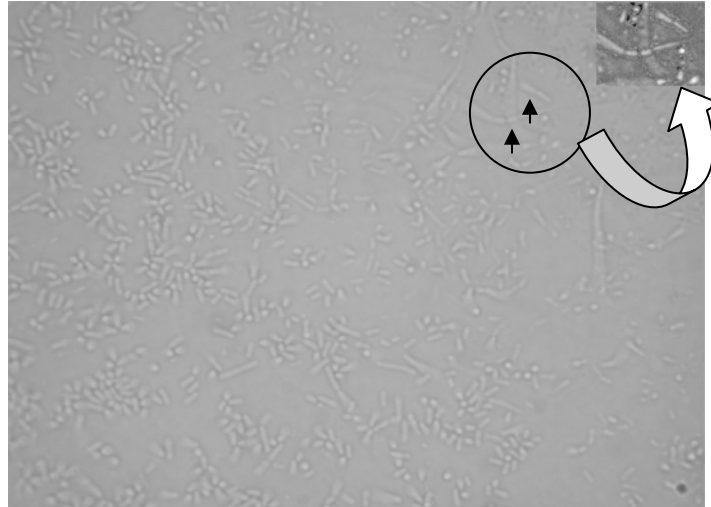


Figure 5.7 Light microscope image depicting cells of CH2B associated with the glass slide surface and chains of cells reaching into the flowing media (arrows) following 24 h of flow cell incubation in nutrient-poor media (EAOB) ($\times 1000$ magnification). Insertion: Enhanced image (Paint Shop Pro v.7) depicting cell-to-cell adherence in cell chains from encircled area

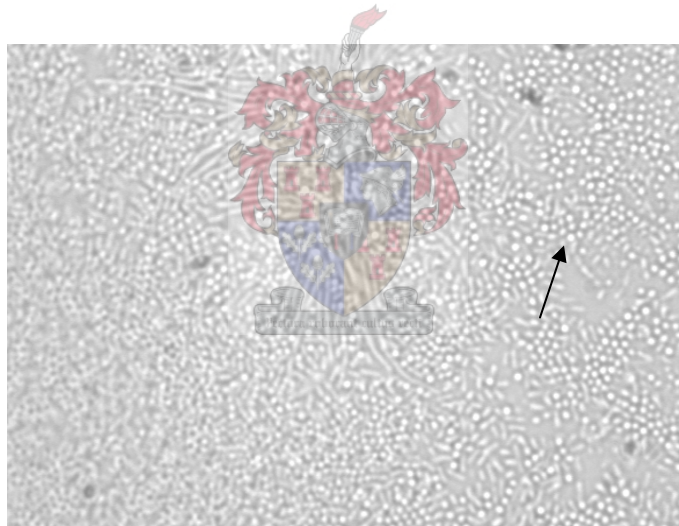


Figure 5.8 Light microscope image depicting polar attachment (arrow) of CH2B cells in microcolony form to glass slide surface and chains of cells reaching from cone-structures into the flowing media (arrow) following 48 h of flow cell incubation in nutrient-poor media (EAOB) ($\times 1000$ magnification).

Isolate CH8 displayed preference for adherence at the outlet side of the flow chamber, as well as at the side of the glass slide where the silicone adhesive and the Perspex met, indicating a preference for hydrophobic surfaces for adherence (Fig. 5.9). After 48 h of incubation, no biofilm formation was observed, confirming the microtitre

plate assay results of this isolate showing weak adherence and biofilm-forming capacity (Table 5.1).

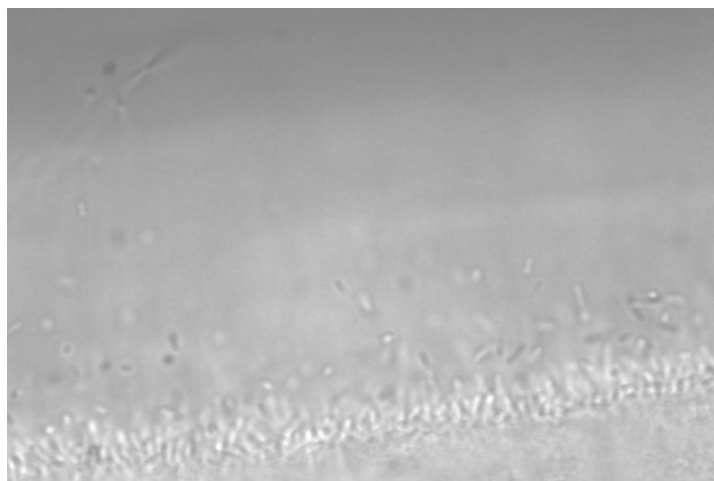


Figure 5. 9 Light microscope image depicting CH8 cells associated with the glass-perspex interface following 24 h of flow cell incubation in nutrient-poor media (EAOB) (\times 1000 magnification).

Isolate CH15 displayed microcolony formation and polar attachment of the cells following 24 hours of incubation, and subsequent cone-formed biofilm structures at 48 hours of incubation in nutrient-poor (EAOB) media. (Fig. 5.10). As with isolate CH8, isolates appeared to adhere more prominently along the edges of the glass slide at the perspex-glass interface.

For study isolates CH23, CH25 and CH26, very few, single cells were observed attached to the glass slide surface after 48 h of incubation in nutrient-poor (EAOB) media. In addition, cells were very thin and short, and a lot of movement was observed. As the study isolates did not possess motility mechanisms, the movement indicated loose attachment with cells being moved rapidly across the glass surface by the flow of the medium.

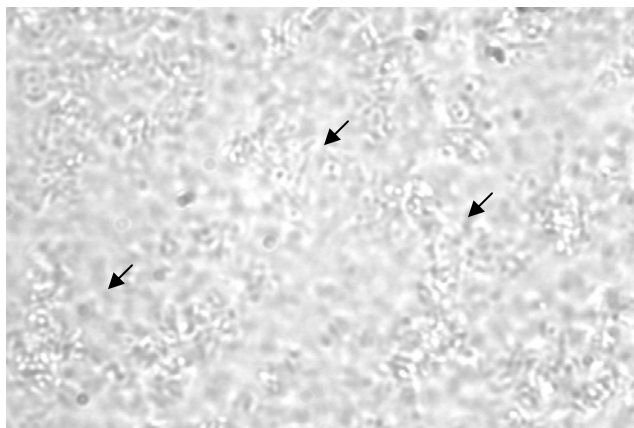


Figure 5. 10 Light microscope image depicting cone formation (arrows) of biofilm structures formed by isolate CH15 on the glass slide surface following 48 h of flow cell incubation in nutrient-poor media (EAOB) ($\times 1000$ magnification).

Of the seven *Chryseobacterium* and *Empedobacter* spp. isolates tested by flow cell studies in nutrient-poor (EAOB) media, isolate CH34 appeared to attach to the glass slide and form biofilms most rapidly. Biofilm formation in the form of cone-like biofilm structures was observed within 24 h of incubation in EAOB. However, after 48 h of incubation biofilm-formation was not as complex as observed for isolates CH2B and CH15. Preferential attachment was observed to take place at the inlet side of the flow chamber. Majority of isolate CH34 cells attached to the glass surface along their length, and polar attachment was less frequent compared to the other study isolates (Fig. 5.11).

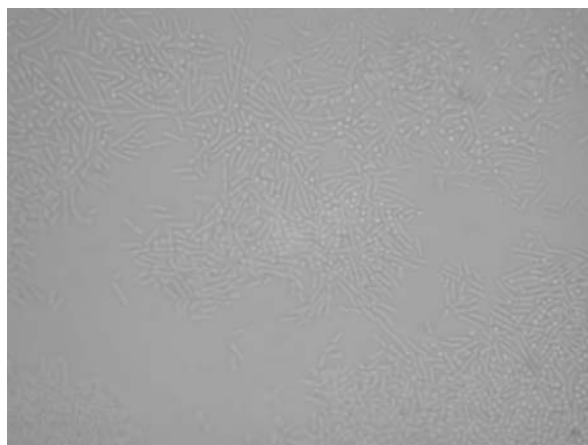


Figure 5. 11 Light microscope image depicting cells of isolate CH34 associated with the glass slide surface following 24 h of flow cell incubation ($\times 1000$ magnification).

In nutrient-rich media (TSB), *Myroides* and *Empedobacter* spp. isolate MY1 did not display as prolific biofilm structures as observed in nutrient-poor (EAOB) media. After 24h and 48 h of growth, only monolayers of cells were observed on the glass surface, and the majority of the cells displayed polar attachment (Fig. 5.12).

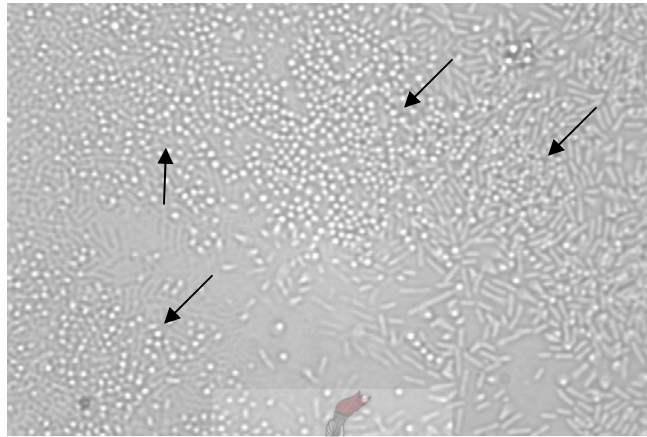


Figure 5. 12 Light microscope image depicting polar attachment (arrow) of MY1 cells in nutrient-rich (TSB) media following 48 h of flow cell incubation ($\times 1000$ magnification).

For the *Chryseobacterium* and *Elizabethkingia* spp. isolates CH2B and CH15, similar results were obtained in nutrient-rich (TSB) flow cell experiments. After 24 h of incubation, overall initial attachment of cells on the glass slides was observed. Cells were mostly attached along their length, with few long single cell chains reaching into the media (Figs. 5.13-5.14). Biofilm structures resembling cone-like structures were observed from a few microcolonies after 48 h of incubation, but the structures observed were not as complex as those observed in nutrient-poor conditions (Figs. 5.15-5.16).

Cells of isolate CH8 appeared to adhere mostly to the inlet side of the flow cell chamber. Cell sizes varied from filamentous, long rods to shorter rods in microcolonies scattered over the surface after 24 h of growth (Fig. 5.17). Although microcolonies increased in size after 48 h, no biofilm formation was observed (Fig 5.18).

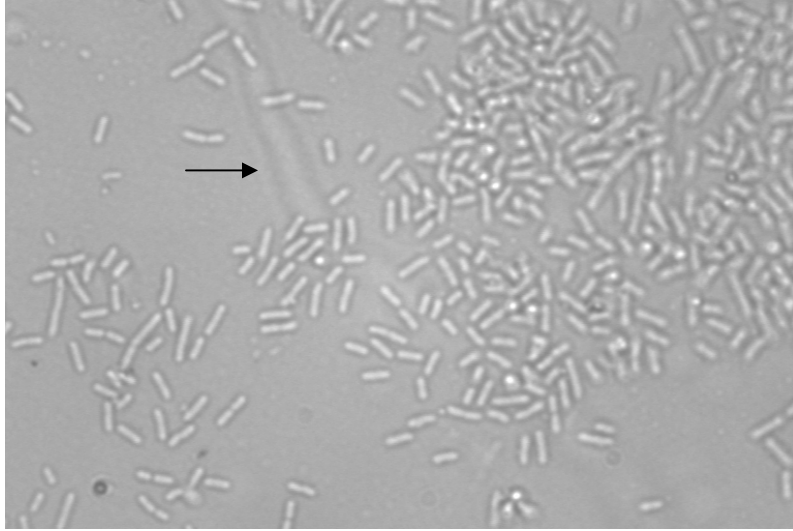


Figure 5.13 Light microscope image depicting isolate CH2B cells associated with the glass slide surface and chains of cells reaching into the flowing media (arrow) following 24 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

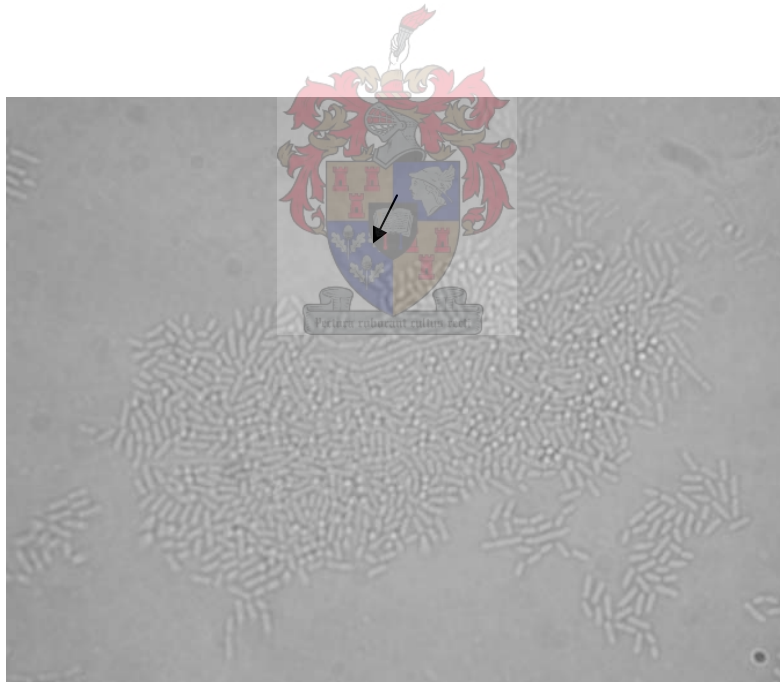


Figure 5.14 Light microscope image depicting isolate CH15 cells associated with the glass slide surface and chains of cells reaching into the flowing media (arrow) following 24 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

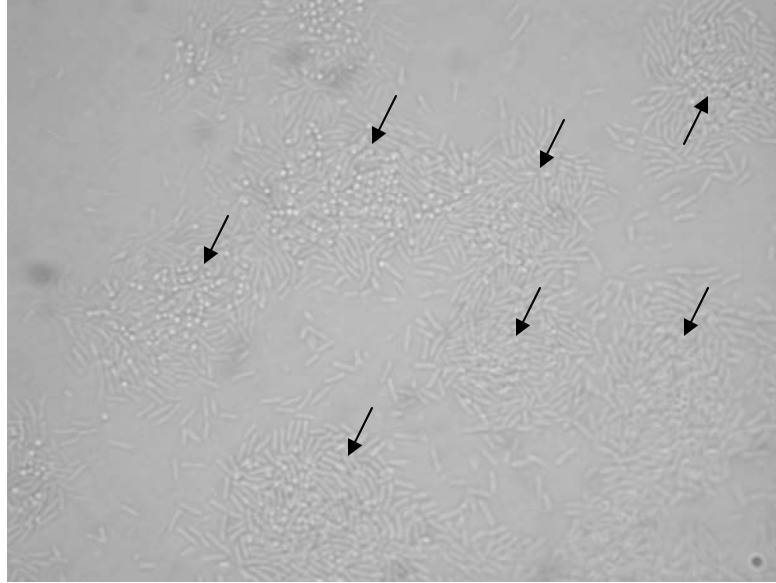


Figure 5. 15 Light microscope image depicting isolate CH2B cells associated with the glass slide surface as microcolonies displaying simple biofilm structures (arrows) following 48 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

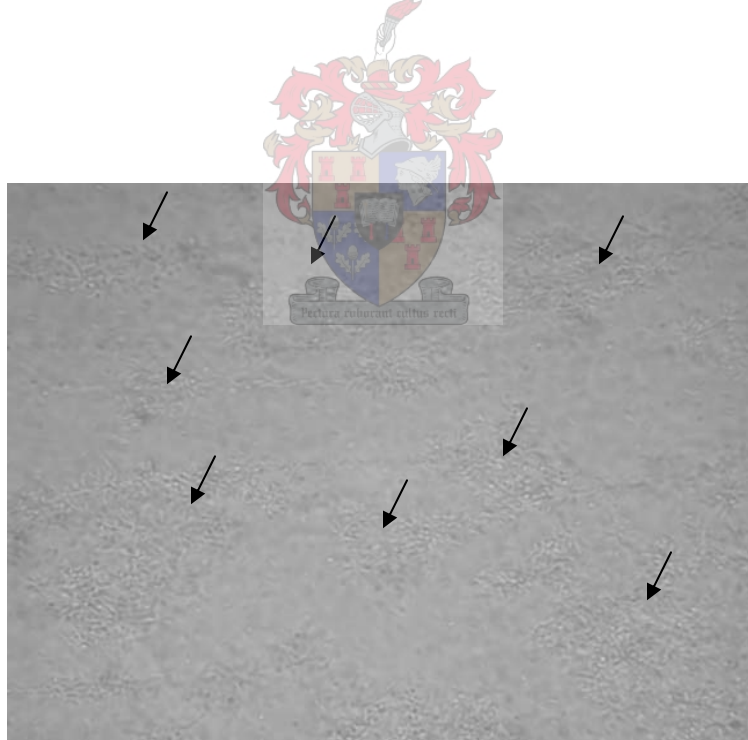


Figure 5. 16 Light microscope image depicting isolate CH15 cells associated with the glass slide surface as microcolonies displaying simple biofilm structures (arrows) following 48 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

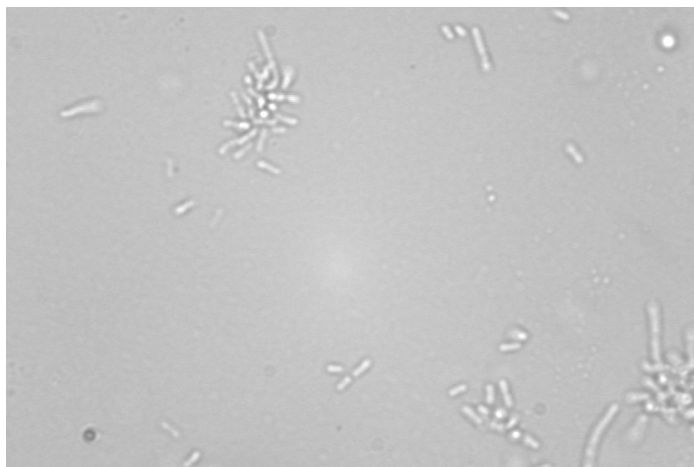


Figure 5. 17 Light microscope image depicting scattered microcolonies of isolate CH8 cells associated with the glass slide surface following 24 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

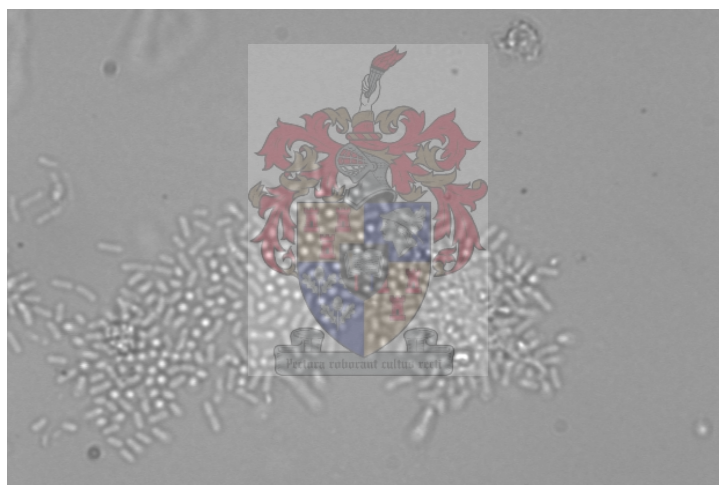


Figure 5. 18 Light microscope image depicting larger microcolonies of isolate CH8 cells associated with the glass slide surface following 48 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

For study isolates CH23 and CH25, adherence to the glass surface by a few single cells was observed. Cells of isolate CH23 did not display adherence after 24 h and this remained unchanged in the next 24 h (Fig. 5.19).

Attachment of single cells only occurred following 48 h of incubation by isolate CH25 cells (Fig. 5.20). Cells of both isolates CH23 and CH25 appeared to be twitching and rotating in place. Although the cells of isolate CH26 appeared not to adhere to glass

surfaces, a cotton-like assemblage was observed at the inlet side of the flow cell chamber. Similar cotton-like growth was observed for isolate CH34 throughout the flow chamber. Although chains of cells were observed, no attachment of cells to the glass surface was noticeable, and growth seemed more prominent at the bottom of the chamber.

Cell-counts on viable bacterial cells collected from the waste/effluent washout of the flow cell system ranged from 8.7×10^{10} cells.ml⁻¹ to 3.95×10^{13} cells.ml⁻¹ in nutrient-poor (EAOB) media, and 1.46×10^{11} cells.ml⁻¹ to 1.8×10^{13} cells.ml⁻¹ in nutrient-rich (TSB) media following 24 hrs of incubation.

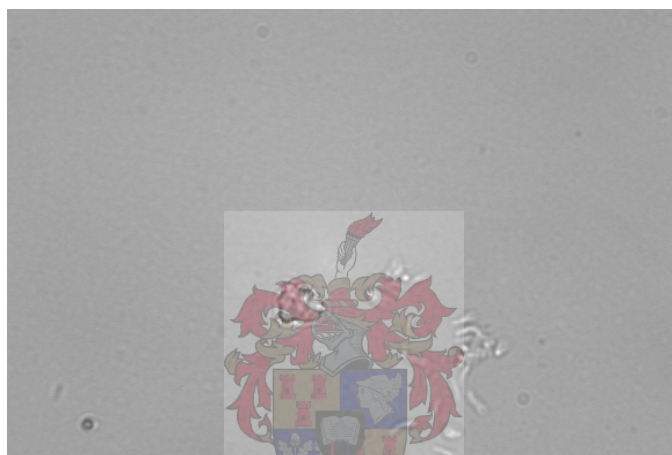


Figure 5. 19 Light microscope image depicting isolated single cells of isolate CH23 associated with the glass slide surface following 24 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

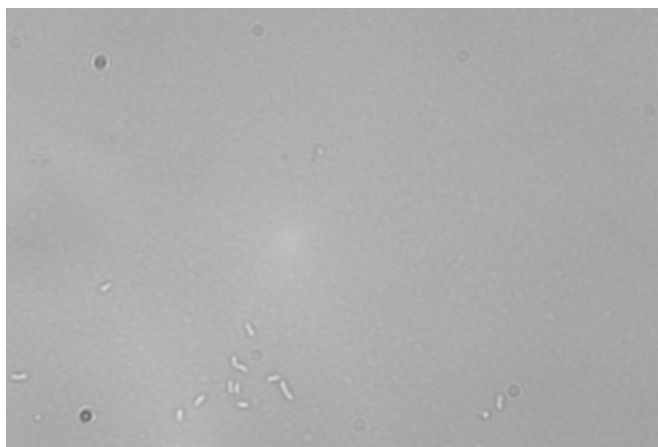


Figure 5. 20 Light microscope image depicting loosely attached, scattered single cells of isolate CH25 associated with the glass slide surface following 24 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

5.3.2 Pure culture, multi-species flow cell experiments

Distinction between bacterial strains in mixed-culture experiments was made visually by comparing images to that of pure culture, single-species flow cell experiments. Cells differed morphologically, with *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. being longer, thinner cells, and *Listeria* spp. short, thick cells.

When co-inoculated into flow cell chambers containing nutrient-poor (EAOB) media with *L. monocytogenes*, isolate MY1 cells scattered across the glass surface after 24 h of inoculation (Fig. 5.21). After 48 h, it appeared as if the *L. monocytogenes* cells formed microcolonies underneath a carpet of MY1 cells which covered the glass surface (Fig. 5.22). In nutrient-rich (TSB) media, a similar pattern of surface colonization by the MY1 cells was observed with either microcolonies (Fig. 5.23) formed within 24 h, or filamentous ‘rope-like’ structures (Figs. 5.24-5.25) of *L. monocytogenes* formed under the monolayer of MY1 after 48 h of growth.

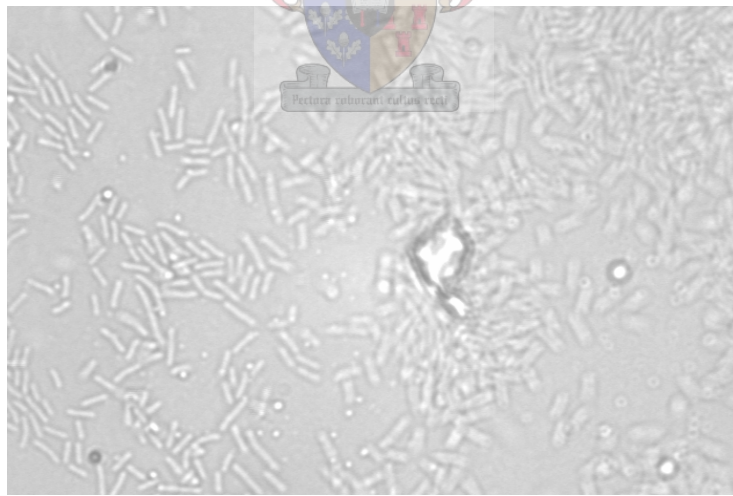


Figure 5. 21 Light microscope image depicting adherence of isolate MY1 cells associated with the glass slide surface following 24 h of flow cell incubation in nutrient-poor (EAOB) media, when co-inoculated with *L. monocytogenes* ($\times 1000$ magnification).

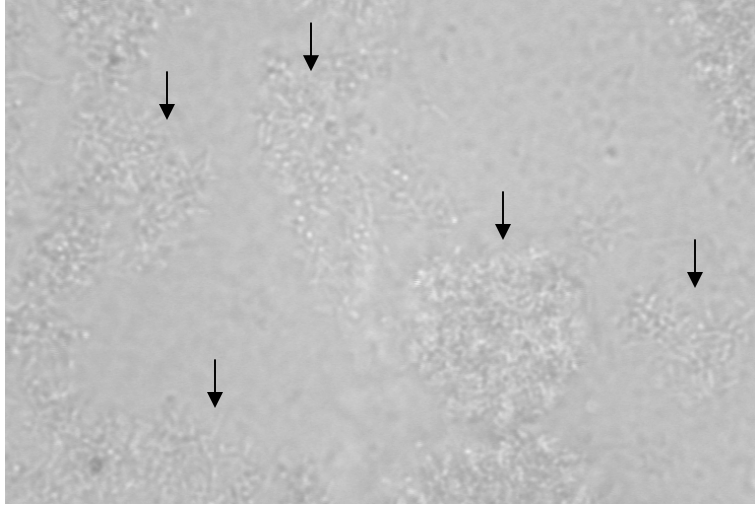


Figure 5. 22 Light microscope image depicting microcolonies of *L. monocytogenes* (arrows) below a carpet of MY1 cells following 48 h of flow cell incubation in nutrient-poor (EAOB) media ($\times 1000$ magnification).

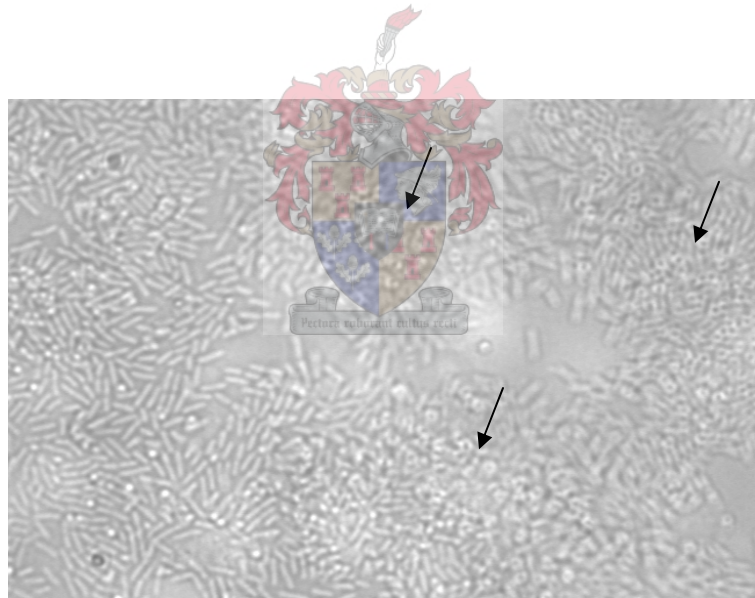


Figure 5. 23 Light microscope image depicting microcolonies of *L. monocytogenes* (arrows) below a carpet of MY1 cells following 48 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

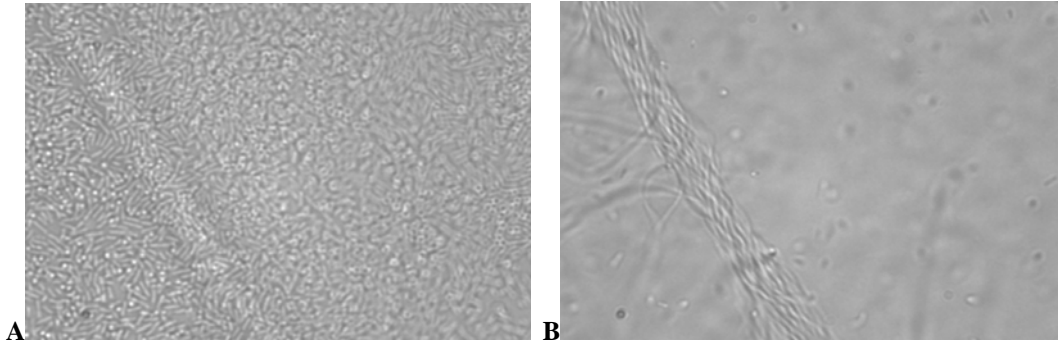


Figure 5.24 Light microscope image depicting the carpet of MY1 cells covering the surface of the glass slide (A) and rope-like filamentous structures of *L. monocytogenes* below the carpet of MY1 cells (B) following 48 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

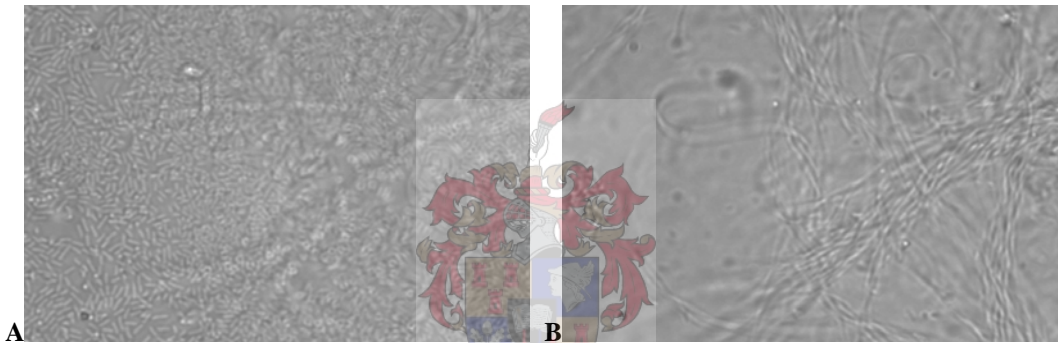


Figure 5.25 Light microscope image depicting the carpet of MY1 cells covering the surface of the glass slide (A) and rope-like filamentous structures of *L. monocytogenes* below the carpet of MY1 cells (B) following 48 h of flow cell incubation in nutrient-rich (TSB) media ($\times 1000$ magnification).

When co-inoculated in nutrient-poor (EAOB) media, both isolate CH2B and *L. monocytogenes* displayed delayed attachment to the glass surfaces, and attached cells were only observed 48 h following inoculation. Although both CH2B and *L. monocytogenes* cells were able to attach to the glass slides, distinct colonies were formed with no association between the different species (Fig. 5.26). In nutrient-rich (TSB) media, cells of both species were scattered over the surface after 24 h of inoculation, although after 48 h only a monolayer of isolate CH2B was observed covering the surface of the glass slide.

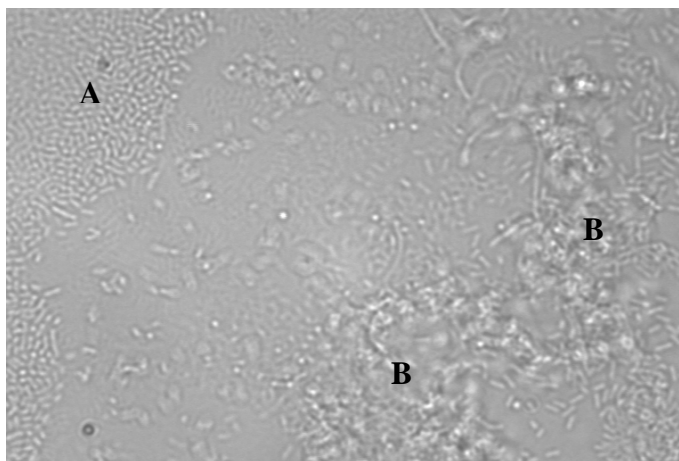


Figure 5. 26 Light microscope image depicting the distinctly separated adherence of CH2B cells (A) and *L. monocytogenes* microcolonies (B) following 48 h of flow cell incubation in nutrient-poor (EAOB) media ($\times 1000$ magnification).

5.4 Discussion

Although the *Myroides* and *Empedobacter* spp. isolate MY1 displayed higher biofilm-forming capacities in nutrient-rich (TSB) media (1.31 – 1.35) than in nutrient-poor (EAOB) media (0.73 – 0.86) to the hydrophobic polystyrene surfaces in microtitre adherence assays (section 3.3.1), better biofilm-formation was observed to hydrophilic glass surfaces in nutrient-poor (EAOB) media during flow cell studies. Surface colonization and the formation of cone-like biofilm structures was observed for this isolate within the first 24 hrs of incubation, which was consistent with the results obtained in the time course of adherence assay where best adherence was determined at 16 h (section 3.3.1), although, no drastic decrease in biofilm formation or adherence was observed after 48 h of incubation as observed in the adherence assays (Fig. 3.5; section 3.3.1). The cone-like architecture of MY1 biofilm structures narrowing toward the flowing media and forming long single-cell chains, may be due to the influence of shear force or increased hydrodynamic conditions of the flowing media in the centre of the flow cell chambers. This architecture has previously been described for *P. aeruginosa* biofilms under high flow rates of an aqueous medium (Stoodley *et al.*, 1999). It was found that under high shear forces in fast flowing aqueous media, biofilm cell clusters of *P. aeruginosa* became elongated in the downstream direction of unidirectional flow to

form filamentous streamers, which became elongated over time, with the tails getting thinner until there was only a small chain of single cells at their tips (Stoodley *et al.*, 1999). The *Myroides* spp. isolates displayed a very hydrophilic surface hydrophobicity with both SAT and BATH hydrophobicity assays (section 3.2.2); and this hydrophilicity may be implicated in the strong adherence and great affinity for the hydrophilic glass surface used in the flow cell system.

It has been found that the attachment of *Listeria monocytogenes* to stainless steel food surface areas was largely increased by the presence of *Myroides* spp. strains (Bremer *et al.*, 2001). In section 3.3.11 it was proposed that inducing molecules could be produced by the MY1 isolate, as no significant induction of adherence of this isolate was observed in the presence of *L. monocytogenes*. Mixed-species flow cell studies revealed layered attachment of these two species, with MY1 attached to the surface and *L. monocytogenes* forming microcolonies or filamentous structures beneath the surface covering layer of MY1 cells. Consistent with the coaggregation studies (section 4.3.2) which indicated a low index of coaggregation between the two species, it was observed that although the species were able to co-exist in a biofilm community, no intimate association between the cells was observed. Although the autoaggregation index for MY1 was not very high, autoaggregation between the cells could be the reason for association and aggregation of the cells in nutrient-poor (EAOB) media, leading to bridge formation by these cells in the higher hydrodynamic conditions of the flow cell chamber.

Microscopic analysis of biofilm formation using flow cells revealed important qualitative characteristics and differences displayed by the *Chryseobacterium/Elizabethkingia* spp. isolates. Biofilms formed by each of the seven study isolates differed in terms of structure and morphology, as well as nutrient condition preference. Biofilm structures ranged from small micro-colonies formed by isolate CH8 in nutrient-rich (TSB) media to multi-layered biofilms with intricate cone-like structures formed by isolates CH2B, CH15 and CH34 in nutrient-poor (EAOB) media.

Isolate CH2B displayed stronger adherence to polystyrene surfaces in microtitre adherence assays in nutrient-rich (TSB) media (BFC – 2.56 to 3.84) than in nutrient-poor (EAOB) media. (BFC – 0.92 to 1.22), although more complex biofilm structures were observed in nutrient-poor (EAOB) media flow cell systems. In nutrient-poor (EAOB)

media, majority of the cells attached by their polar sides which could be an attempt to increase surface area for nutrient uptake in nutrient-limited environments, since horizontal attachment was observed in nutrient-rich (TSB) media. Conversely, this attachment could also be a looser attachment than that observed for nutrient-rich (TSB) media, and thus indicate attachment to surfaces for a shorter period in nutrient-limited environments.

A very hydrophilic nature was obtained for isolate CH2B with the BATH (0.8 %) and SAT assays, and this was noticeable in the flow cell studies as the cells adhered along the entire surface of the glass slide. Cells of isolate CH2B formed similar single cell chains as observed in the MY1 biofilm structure. Isolate CH2B displayed an autoaggregation index of 37 % (section 4.3.1) which could explain the aggregation of these cells in the high shear area of the flow-cell chamber.

In the mixed-species flow cell experiment, both CH2B and *L. monocytogenes* attached to the glass slide, although the high coaggregation displayed by these two bacterial species (section 4.3.2) was not apparent, as the microcolonies of the two species formed apart from one another on the glass surface.

Cone-like biofilm structures were also observed for isolates CH15 and CH34 in the flow cell experiments. Isolate CH15 formed more complex structures in nutrient-poor (EAOB) media although higher biofilm-forming capacity values in the microtitre assay were obtained for this isolate in nutrient-rich (TSB) media. As with isolate CH2B, isolate CH15 cells displayed polar attachment in nutrient-poor (EAOB) media, and horizontal attachment in nutrient-rich (TSB) media. The moderate hydrophobicity of 41% obtained in the BATH assay for CH15 cells might explain the preference of these cells for adherence to the glass-perspex interface. Isolate CH34 adhered and formed biofilm structures within the first 24 h of incubation with adherence and biofilm formation being more complex at the inlet side of the flow cell chambers. This is consistent with the time course of adherence results indicating adherence within 16 h of incubation. It was also observed that adherence of isolate CH34 cells was consistent in both nutrient types, as observed in microtitre adherence assays, although adherence to the perspex bottom of the chamber was more prominent in nutrient-rich (TSB) media. Similarly, isolate CH26 formed a mucoid clump of cells at the inlet side of the chamber

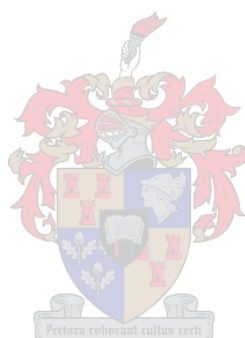
in nutrient-rich (TSB) media, although no surface attachment was observed for these cells during the flow cell experiments. No biofilm structures were observed for isolate CH8, as with isolate CH15, adherence was observed at the glass-perspex interface. Surface hydrophobicity for isolates CH8, CH26 and CH34 was determined as very hydrophilic by the BATH assay with percentages of 14, 9 and 13 %, respectively and surface hydrophobicity cannot, therefore, be the only factor influencing glass-perspex interface adherence these isolates. This preference could be attributed to difference in texture, such as roughness of the perspex sides which has been shown to increase the adherence abilities of *Y. ruckeri* to fish farm materials (Coquet *et al.*, 2002a). It was also observed that adherence was more prominent at the outlet side of the flow cell chamber, which could indicate a series of changes in the cell surface composition before adherence was induced. Although isolate CH25 displayed a strong adherence profile in nutrient-poor (EAOB) media, during adherence assays (BFC – 3.5 to 3.7), this isolate together with the non-biofilm forming isolate CH23 did not display biofilm formation in the flow cell experiments, suggesting that shear rates might impact the ability to form biofilm structures.

Sampling of the effluent revealed $>10^{10}$ cells.ml⁻¹, suggesting that biofilms function as a source of planktonic cells through high cell yield and detachment. In a previous study the planktonic-cell yield of a *Pseudomonad* biofilm was found to be $>10^9$ cells.ml⁻¹ (Bester *et al.*, 2005).

The present study has shown the strong biofilm-forming ability of *Myroides* and *Empedobacter* spp. isolate MY1, and the strain-to-strain variation in the ability of *Chryseobacterium* and *Elizabethkingia* spp. isolates to form biofilms. Hydrophobicity characteristics of these organisms could not be consistently correlated with the capacity to form biofilms, and initial adherence abilities could not be correlated to biofilm formation in flow cell experiments. Nevertheless, it was evident that members of these genera possess the ability to colonize and form intricate biofilm structures in hydrodynamic environments simulating the natural aquaculture environment from which they were isolated. Co-existence in biofilm communities with the food pathogen *L. monocytogenes* was also observed not only for the *Myroides* and *Empedobacter* spp.

isolate, but also for the *Chryseobacterium* and *Elizabethkingia* spp. isolate. However, the nature and degree of this relationship remains to be elucidated.

Multi-species interactions demonstrated by these organisms displays the importance of biofilm formation studies by these opportunistic fish pathogens, since association with aquacultural, veterinary and human pathogens, as well as food-spoilage organisms could lead to infection and re-infection of aquaculture live-stock and subsequent spoilage of aquaculture food products, and alarmingly a horizontal spread of MDR phenotypes from these isolates to medically-important bacterial species.



CHAPTER SIX

Concluding Remarks

6.1 The Research in Perspective

In the present study, *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolated from a variety of fish species in South Africa were identified and characterized by a combination of phenotypic and biochemical methods. The ability of these isolates to adhere to surfaces, and their biofilm-forming capacity was investigated with adherence assays and a flow cell system simulating their natural environmental conditions. In addition, the capacity of these isolates to form biofilms was investigated by studies of cell-surface properties which potentially play a role in adherence to substrates. The association of the isolated species with various bacterial species important in the aquaculture, medical and food industries was investigated to elucidate the organization of the study isolates and other bacterial species in natural mixed-species biofilm communities. This is the first report on the investigation of the adherence- and biofilm-forming capacities of yellow-pigmented *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* from aquaculture systems in South Africa.

Phenotypic, physiological and biochemical characterization allowed limited discrimination of the 5 *Myroides* and *Empedobacter* spp. and 34 *Chryseobacterium* and *Elizabethkingia* spp. isolates. This is in accordance with other studies based on the identification and characterization of *Chryseobacterium* and *Elizabethkingia* spp. isolates (Bernardet *et al.*, 2005) and *Myroides* and *Empedobacter* spp. isolates (Hugo *et al.*, 2005; and Vancanneyt *et al.*, 1996). However, these methods are essential for preliminary identification of *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates to the genus level, and were thus useful in the current study (Bernardet *et al.*, 2006; Bernardet *et al.*, 2005; and Hugo *et al.*, 2005).

16S rRNA gene PCR-RFLP, RAPD fingerprinting, and WCP analysis did not allow the discrimination of the *Myroides* and *Empedobacter* spp. isolates, although discrimination between the isolates was observed with OMP analysis (Discrimination

index – 0.53). The *Myroides* and *Empedobacter* spp. isolates could therefore not be characterized to species level using the molecular typing techniques used in the current study.

Discrimination of the *Chryseobacterium* and *Elizabethkingia* spp. isolates with 16S rRNA gene PCR-RFLP using restriction enzymes *CfoI* and *TaqI*, RAPD fingerprinting with primer P1 and WCP analysis was limited (Table 2.10), although molecular typing of these isolates using 16S rRNA gene PCR-RFLP using restriction enzyme *TaqI*, RAPD fingerprinting with primer P2 and OMP analysis, provided evidence of the genetic heterogeneity among closely related *Chryseobacterium* and *Elizabethkingia* spp. isolates (Table 2.10). The discriminatory power of RAPD fingerprinting was demonstrated by Bernardet *et al.* (2005) who observed delineation of clusters of species belonging to the genus *Chryseobacterium* through RAPD analysis, although the discrimination with WCP analysis obtained by Bernardet *et al.* (2005) was not apparent in the current study.

The limited discrimination power of 16S rRNA gene PCR-RFLP could be due to 16S rRNA gene sequence similarities since up to 99% similarity has been described between *Chryseobacterium* spp. (Li *et al.*, 2003). It indicates the overall high similarity, and presents the difficulty in delineating species belonging to this genus using this typing technique. Study isolates could not be clustered with any of the reference strains with certainty, possibly due to adaptational differences to the environments from which they were isolated and therefore, none of the isolates could be characterized to species level. The presence of common bands in profiles obtained with RAPD primers P1 and P2 could potentially be used to identify genes or gene sequences which will allow rapid molecular-based identification of *Chryseobacterium* and *Elizabethkingia* species (diagnosis) or their species group (species assignment).

High MAR indices were obtained for the *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates obtained from Blue tilapia aquaculture systems that had not previously been exposed to antibiotic treatment. These results indicated contamination from environments where the isolates were exposed to antibiotic agents, or that these isolates acquired the resistance determinants through horizontal gene transfer within the aquaculture system. Such high antibiotic resistance

phenotypes raise concern regarding antibiotic treatment of aquaculture disease outbreaks, and the spread of these opportunistic pathogens to environments where they could infect immunocompromised humans. In addition, the spread of antibiotic resistance genes in biofilm communities has been reported (Harrison *et al.*, 2005) and thus horizontal gene transfer of antibiotic resistance genes from these isolates to pathogenic organisms in aquaculture systems or potable water systems could lead to major economic losses in the aquaculture industry and uncontrollable disease outbreaks in humans.

The biofilm-forming ability of *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates was confirmed by means of microtitre-plate adherence assays and flow cell studies. Both techniques effectively differentiated strong and weakly adherent isolates. The modified Stepanovic *et al.* (2000) microtitre-plate adherence assay was a rapid and highly reproducible technique for screening a large group of isolates. Application of the equation for the calculation of biofilm-forming capacity (van Houdt *et al.*, 2004) allowed for correlation between biofilm formation and relative biofilm-forming capacity in order to accurately determine the biofilm-forming isolates. Although the flow cell study was more time-consuming, important qualitative information about the biofilm structures produced by the study isolates was obtained.

The *Myroides* and *Empedobacter* spp. isolates displayed strong adherence and high relative biofilm-forming capacity. The impact of environmental cues, i.e., temperature, nutrient availability, and hydrodynamic conditions, was apparent in these assays as the isolates displayed a preference to adhere at lower temperatures (~21 °C) and nutrient-rich environments with low hydrodynamic forces (static incubation). It was also observed that these isolates were capable of moderate adherence in higher temperatures (37 °C), indicating their ability to adhere in warm-blooded animal hosts. The effect of cell-surface properties on adherence, such as the qualitative determination of thick capsular material surrounding the cells, and very hydrophilic nature of the cell surface as determined by both SAT and BATH hydrophobicity assays, could not be correlated to the adherence of the *Myroides* and *Empedobacter* spp. isolates to polystyrene surfaces. In flow-cell systems, the selected *Myroides* and *Empedobacter* spp. isolate displayed typical 'cone-like' biofilm structures, intricate bridge formation between microcolonies and a

strong affinity for hydrophilic glass surfaces for biofilm formation, correlating to the very hydrophilic nature as was observed in the hydrophobicity assays. The effect of increased hydrodynamic conditions on the biofilm architecture was also observed as the biofilm structures were observed to narrow toward the increased dynamic areas forming single-cell chains as previously described for other bacterial species (Rickard *et al.*, 2004b; and Stoodley *et al.*, 1999).

Although variation in the biofilm-forming capacities was observed for the *Chryseobacterium* and *Elizabethkingia* spp. isolates, the impact of environmental cues on the adherence abilities of the isolates was also evident, as majority of these isolates preferred to adhere at ambient temperature (~21 °C), under low hydrodynamic conditions (static incubation) in nutrient-rich environments. It was, however, observed that some of the isolates displayed strong adherence at 37 °C, indicating the diversity of environments *Chryseobacterium* and *Elizabethkingia* spp. isolates are able to colonise. The presence of molecules produced by other bacterial species which possibly include quorum sensing molecules was found to play a role in the induction of increased biofilm formation by isolates capable of weak adherence prior to induction. It is, therefore, likely that weakly adherent *Chryseobacterium* and *Elizabethkingia* spp. isolates may be incorporated into biofilms more readily in the presence of other bacterial species, or their extracellular products and contribute to an overall increased risk of medical and veterinary infection and disease, secondary infection in the aquaculture environment, spoilage of food products as well as industrial or potable water system contamination.

The qualitative observation of expression of a capsule layer by majority of the *Chryseobacterium* and *Elizabethkingia* spp. isolates could not be correlated to the different adherence abilities of the isolates. In addition, no correlation was observed between hydrophobicity profiles obtained by the BATH assay. Increased hydrophobicity determined by the SAT assay, however, could be correlated to increased adherence of the isolates at ambient temperature in nutrient-poor conditions under dynamic/static incubation, as well as at 37 °C in nutrient-rich media under dynamic conditions. In accordance to previous findings (Basson *et al.*, in press; Ofek *et al.*, 2003; and Babelona *et al.*, 2001), no correlation could be found between the hydrophobicity profiles determined by the SAT and BATH assays in the present study.

Chryseobacterium and *Elizabethkingia* spp. isolates selected for investigation of biofilm formation in the flow-cell system, displayed variation in their adherence abilities from non-adherent to strongly adherent in the microtitre adherence assays. Microscopic analysis of biofilm formation in flow cell systems revealed important qualitative characteristics and differences displayed by the study isolates. Biofilm formation differed between study isolates in terms of structure and morphology, as well as nutrient condition preference. Biofilm structures ranged from small micro-colonies to multi-layered biofilms with intricate 'cone-like' structures, and the effects of hydrodynamic conditions were also observed in these biofilm structures. For some of the isolates, polar attachment of the cells were observed in nutrient-limited conditions as opposed to horizontal attachment in nutrient-rich environments indicating that the adherence behaviour of the isolates is influenced by nutrient availability and that these organisms may be able to regulate attachment/detachment in response to nutrient conditions, or regulate surface exposure so as to increase nutrient uptake when conditions are limited.

Initial adherence abilities of the isolates could not be directly correlated to their ability to form biofilms, although this could be due to the differences in surface hydrophobicity of the polystyrene surfaces used in microtitre adherence which is hydrophobic, and the hydrophilic glass surfaces used in the flow cell assay. For the strongly adherent and biofilm-forming isolates included in the flow cell experiments, it was observed that the isolate (CH2B) displaying very hydrophilic surface hydrophobicity values with the BATH and SAT assay associated better with the glass surface, whereas the isolate displaying more hydrophobic properties (CH15) in the BATH and SAT assays adhered more readily to the glass-perspex interface of the flow cell chambers. However, a consistent trend relating to hydrophobicity determined by BATH and SAT assays and adherence preference to either glass or the glass-perspex interface was not observed for all the isolates included in the flow cell study, and it is thus proposed that hydrophobicity is not the sole factor influencing adherence preferences of these isolates.

Association of the *Myroides* and *Empedobacter* spp. and *Chryseobacterium* and *Elizabethkingia* spp. isolates with genetically identical and genetically distinct bacterial strains was investigated by autoaggregation and coaggregation assays. *Myroides* and *Empedobacter* spp. isolates displayed variation in autoaggregation indices, correlating to

the visual groupings obtained in the OMP analysis, and thus indicating a possible relationship between the additional OMP present in some of the isolates and the recognition of genetically identical bacterial strains. In addition, it was also found that increased autoaggregation correlated to increased adherence by these isolates in nutrient-rich environments at ambient temperature under dynamic conditions. Diverse autoaggregation indices were also obtained for the *Chryseobacterium* and *Elizabethkingia* spp. isolates. In pure culture flow cell studies, isolates displaying high autoaggregation indices displayed the formation of single cell chains under increased hydrodynamic conditions, which is in accordance to what has previously been described (Rickard *et al.*, 2004b).

High coaggregation indices were observed between the *Myroides* and *Empedobacter* spp. isolates and *E. faecalis* and *S. aureus*. Due to the high overall coaggregation between majority of the coaggregating partners and the isolate displaying the presence of an additional OMP selected for the coaggregation assays, it was suggested that these proteins may be involved in recognition or aggregation of both genetically distinct/identical bacterial strains. Strongest coaggregation was observed between the *Chryseobacterium* and *Elizabethkingia* spp. isolates and *E. faecalis*, *S. enterica* serovar Arizonae, *S. aureus* and *Listeria* spp. coaggregating partner strains. Observations in mixed-species biofilm flow cell experiments could be correlated to results obtained by coaggregation assays. Isolates displaying lower coaggregation indices (*Myroides* spp. isolate MY1 and *Listeria* spp. coaggregation partner strains) appeared to form layered biofilms with no intimate association between the two bacterial strains. In contrast, isolates displaying higher coaggregation indices (*Chryseobacterium* and *Elizabethkingia* spp. isolate CH2B and *Listeria* spp. coaggregation partner strains) were found to simultaneously attach to the glass surface and form mixed colony biofilm structures.

In conclusion, the present study has shown that members of the genera *Myroides*, *Empedobacter*, *Chryseobacterium* and *Elizabethkingia* isolated from South African aquaculture systems possess the ability to adhere to surfaces and form biofilms under various environmental conditions. These isolates displayed the ability to coaggregate with bacterial species important from a food and health perspective, and displayed high

antibiotic resistance profiles. Although they are mostly described as opportunistic pathogens in both veterinary and human infections, their association with pathogens and spoilage organisms causing great economical losses in the aquaculture and food industries and lethal infections in humans raises concern with regards to being associated with potential reservoirs for infection and re-infection as well as their ability to incorporate food spoilage organisms into biofilms with increased cleaning and sanitation resistance on food preparation surfaces.

6.2 Future prospects

Recommended future investigations originating from preliminary findings in the current study would include 16S rRNA sequencing for the possible identification/characterization to species-level of specific *Myroides*, *Empedobacter*, *Chryseobacterium* and/or *Elizabethkingia* isolates. Further investigation and identification of common bands in profiles obtained with RAPD primers P1 and P2, is also recommended as this could potentially be used in the rapid molecular identification of specific *Chryseobacterium* and *Elizabethkingia* species (diagnosis) or their species group (species assignment). An in-depth study on the genes conferring resistance to antimicrobial agents by these isolates is also proposed so as to elucidate the MDR potential of these organisms. Quantitative characterization of the capsular material observed for the study isolates might provide valuable information to the capsules' roles in the adherence abilities of these species. Further investigation into the nature and effect of the OMP observed in the *Myroides* and *Empedobacter* spp. isolates might reveal the role of this protein in the hydrophilicity, and recognition or aggregation of genetically identical as well as genetically distinct bacterial strains by these organisms. Characterization of possible quorum sensing molecules involved in induction of the adherence of the study isolates is also suggested, for better elucidation of interaction between the study isolates and other bacterial strains in biofilm communities, as well as the effect of study isolates on important bacterial strains, or *visé versa*. Furthermore, the investigation of specific cell-surface molecules mediating strong coaggregation abilities between the study isolates and coaggregating partners may provide valuable information

for anti-adhesion therapy which could be applied in aquaculture systems for the eradication of biofilms harbouring pathogenic organisms.



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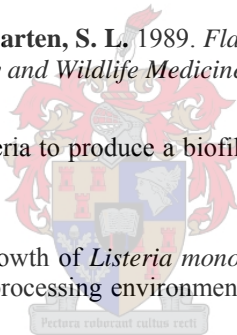
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