COMPARATIVE PROTEOMIC AND GENOMIC ANALYSIS OF *Flavobacterium johnsoniae*-like BIOFILM, PLANKTONIC AND AGAR SURFACE-ASSOCIATED CELLS

By

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DECLARATION

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OPSOMMING

Patogene *Flavobacterium* spp. veroorsaak ernstige infeksie uitbrake in ’n verskeidenheid gekweekte vissoorte, wat jaarliks tot groot ekonomiese verliese in die akwakultuur bedryf lei. Die vermoë van *Flavobacterium johnsoniae*-tipe isolate om as oppervlak-gehegde gemeenskappe (biofilms) in akwakultuur sisteme te groei bedreig visgesondheid oor verlengde periodes. Die vermoë van 28 *F. johnsoniae*-tipe isolate om biofilms te vorm is vergelyk met hul vermoë om chitien te degradeer, die profiel van hul ekstrasellulêre koolhidraat komplekse (EKK) en bandpatrone verkry met puls-veld jel elektroforese (PVJE). Fisiologiese veranderinge in die proteoom van 5-dag-oue planktoniese-, biofilm- en agar oppervlak-geassosieerde kulture van *F. johnsoniae*-tipe isolate YO12 en YO64 is met twee-dimensionele (2-D) jel elektroforese geanaliseer. Sewentien differensieël uitgedrukte en 14 uniek uitgedrukte proteïene is deur middel van matriks-geassisteerde laser desorpsie ioniserings-tyd van vlug-massa spektrometrie (MGLDI-TVV MS) geïdentifiseer. Twee-en-dertig differensieël uitgedrukte gene in 5-dag-oue biofilm- en agar oppervlak-geassosieerde kulture van *F. johnsoniae*-tipe isolate YO12 en YO64 was deur middel van suppressie afgetrokke hibridisasie (SAH) geïdentifiseer. Beduidende negatiewe korrelasies is tussen die chitin-degraderings vermoë en EKK en die biofilm-vormings kapasiteit van 24-uur-oue biofilm kulture van *F. johnsoniae*-tipe isolate waargeneem. Resultate verkry met PVJE het die heterogene samestelling van *F. johnsoniae*-tipe isolate uitgewys. ‘n Beduidende positiewe korrelasie is tussen PVJE groeperings en vis gasheer spesie waargeneem. Differensieël en uniek uitgedrukte gene geidentifiseer in die planktoniese-, biofilm- en agar oppervlak-geassosieerde fases is deur middel van 2-D/MS asook differensieël uitgedrukte gene geïdentifiseer in die biofilm en agar oppervlak-geassosieerde fases deur middel van SAH was as betrokke by aanpassing/beskerming, metaboliese prosesse, membraan/vervoer/ beweeglikheid en transkripsie/translasie gekategoriseer. Sover bekend is hierdie die eerste beskrywing van differensieël uitgedrukte gene en geenprodukte van *F. johnsoniae*-tipe isolate afkomstig van geinfekteerde vis in Suid Afrika.

ABSTRACT

Pathogenic *Flavobacterium* spp. cause serious disease outbreaks in a variety of farmed fish, which lead to large economic losses in the aquaculture industry on an annual basis. The ability of *Flavobacterium johnsoniae*-like isolates to grow as surface-associated communities (biofilms) in aquaculture systems poses a threat to fish health over extended periods of time. The biofilm-forming ability of 28 *F. johnsoniae*-like isolates obtained from diseased fish were correlated with their chitin-degrading abilities and extracellular carbohydrate complexes (ECC) and their pulsed-field gel electrophoresis (PFGE) genotypes. Physiological changes in the proteome of 5 day planktonic, biofilm and agar surface-associated cultures of *F. johnsoniae*-like isolates YO12 and YO64 were analyzed by two-dimensional (2-D) gel electrophoresis and 17 differentially expressed and 14 uniquely expressed proteins were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Thirty-two differentially expressed genes in 5 day biofilm and agar surface-associated cultures of *F. johnsoniae*-like isolates YO12 and YO64 were identified using suppression subtractive hybridization (SSH). Significant negative correlations were observed between the chitin-degrading abilities and ECC and the biofilm-forming capacity of 24 h biofilm cultures of *F. johnsoniae*-like isolates. Genetic heterogeneity was displayed by the *F. johnsoniae*-like isolates following PFGE. A significant positive correlation was observed between PFGE types and fish host species. Differentially and uniquely expressed proteins identified in planktonic, biofilm and agar surface-associated phases by 2-D/MS as well as differentially expressed genes identified in the biofilm and agar surface-associated phases by SSH were categorized as being involved in adaptation/protection, metabolic processes, membrane/transport/ motility and transcription/ translation. As far as we know, this is the first report on the characterization of differentially expressed genes and gene products of *F. johnsoniae*-like isolates obtained from diseased fish in South Africa.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The human population is rapidly expanding and natural food and water resources are becoming limited. The commercial demand for fresh meat has led to an increase in the production of fish in aquaculture farming worldwide. A number of fish species are farmed on a large scale commercially, with salmonids (including trout, salmon and charr) being the most popular group of fish farmed globally and probably the most valuable to man (Bader *et al*., 2003; Bernardet and Bowman, 2006; Wakabayashi, 1993).

Aquaculture systems are ideal environments for the growth of bacteria and other microorganisms, which may affect fish and/or human health. Many aquatic pathogens cause serious fish diseases, which lead to tremendous economic losses in aquaculture farming on an annual basis (Bernardet and Bowman, 2006; Coquet *et al*., 2002a; Gavín *et al*., 2003). A wide range of pathogenic bacterial species, both obligate and those regarded as opportunistic pathogens, cause disease outbreaks in farmed trout and other salmonids (Inglis and Hendrie, 1993; Noble and Summerfelt, 1996; Turnbull, 1993; Wakabayashi, 1993).

Infectious diseases of bacterial origin often associated with disease outbreaks in farmed trout include bacterial gill disease (BGD), bacterial cold-water disease (BCD), rainbow trout fry syndrome (RTFS), columnaris disease, furunculosis, enteric redmouth disease (ERM), bacterial kidney disease (BKD) and fin rot (Bernardet and Bowman, 2006; Coquet *et al*., 2002a; Decostere *et al*., 1997; Decostere *et al*., 1999a and b; Madsen and Dalsgaard, 2000; Noble and Summerfelt, 1996). The majority of these diseases, viz., BGD, BCD, RTFS, columnaris disease and fin rot, are caused by members of the genus *Flavobacterium*, whereas *Aeromonas salmonicida*, *Yersinia ruckeri*, and *Renibacterium salmoninarum* are responsible for furunculosis, ERM and BKD, respectively (Bernardet and Bowman, 2006; Coquet *et al*., 2002a; Decostere *et*

al., 1997; Decostere *et al*., 1999a and b; Madsen and Dalsgaard, 2000; Noble and Summerfelt, 1996). These organisms are all regarded as obligate fish pathogens responsible for primary infections in fish. Disease outbreaks caused by such primary infectious agents may lead to more than 70% mortality in fish stocks, such as in the case of RTFS (Bernardet and Bowman, 2006).

Opportunistic pathogens, such as *Flavobacterium johnsoniae*, *Aeromonas hydrophila* and a number of *Pseudomonas* spp., are primarily responsible for secondary infections in fish (Bernardet and Bowman, 2006; Inglis and Hendrie, 1993; Noble and Summerfelt, 1996). Certain opportunistic pathogens, such as *A. hydrophila* and *Pseudomonas aeruginosa*, also cause serious opportunistic infections in humans, especially immunocompromised patients (Gavín *et al*., 2002; Gavín *et al*., 2003; Kirov *et al*., 2004).

Bacteria, including pathogenic sp., are rarely planktonic in the aquatic environment and generally live as surface-associated colonies or communities (Huq *et al*., 2008; Jackson *et al*., 2002b; Johnson, 2007). The survival of pathogenic bacteria in their natural environment may depend on the flexibility of bacterial gene expression, allowing adaptation during rapidly changing conditions (Jefferson, 2004). A particularly important example of bacterial adaptation through differential gene expression is the ability to mediate between the free-living, planktonic state to the biofilm state, i.e., sessile communities suspended in thick extracellular polymeric substances (EPS) (Bell, 2001; Donlan, 2002; Jefferson, 2004; Stickler, 1999; Webb *et al*., 2003). Aquatic pathogenic bacteria, such as *F. johnsoniae*, *Flavobacterium psychrophilum*, *Y. ruckeri*, *Vibrio cholerae*, *A. hydrophila* and *P. aeruginosa*, which are responsible for animal and/or human disease, are well known for their ability to form biofilms (Álvarez *et al*., 2006; Basson *et al*., 2008; Huq *et al*., 2008).

Biofilm growth appears to be the favoured form of growth, since it provides a means of protection against a number of physical and chemical stresses, such as shear forces, UV radiation, oxidative stress, toxic compounds, thermal stress, limited nutrients, host immune defense systems, phagocytosis and predation in the natural environment (Huq *et al*., 2008; Johnson, 2007). Hence, biofilm-

associated infections are extremely difficult to treat, mainly due to widely differing biofilm populations as well as their increased resistance towards antimicrobial agents in the biofilm state (Bayston, 2000; Bell, 2001).

Biofilm populations contaminate industrial pipelines, cooling systems, dental unit water lines, catheters, medical implants, ventilators and aquaculture systems (Basson *et al*., 2008; Hall-Stoodley and Stoodley, 2002). Bacteria colonize surfaces of both biotic and abiotic compounds frequently found in the aquatic environment, viz., chitin, wood, rock, concrete, metal, fiberglass and polyvinylchloride (Coquet *et al*., 2002a; Pruzzo *et al*., 2008). Biofilm growth of pathogenic bacteria present on surfaces in the aquatic environment, including aquaculture tanks, continuously release bacteria into the surrounding environment which may lead to recurrent disease outbreaks over extended periods of time (Coquet *et al*., 2002a and b; Basson *et al*., 2008; Leonard *et al*., 2000).

1.1. BIOFILM PROCESSES

A biofilm can be defined as a surface-attached community of microorganisms, consisting of either a single or multiple microbial species, embedded in a slime-like matrix of EPS in an aqueous environment (Bell, 2001; Huq *et al*., 2008). Biofilms form over time and the developmental process of biofilm growth (Fig. 1.1) can broadly be divided into the following categories, viz., initial cell attachment, which is reversible; irreversible attachment with the production of extracellular polymers; microcolony formation; biofilm maturation or macrocolony formation; and lastly, detachment and dispersal of cells from the surface of the biofilm into the flowing medium, surrounding the biofilm (Hall-Stoodley and Stoodley, 2002; Van Houdt and Michiels, 2005).

The term *biofilm processes* refers to all the physical, chemical and biological processes taking place in a biofilm system, which affects (or are affected by) the degree of biofilm deposit and expansion and/or the microbial

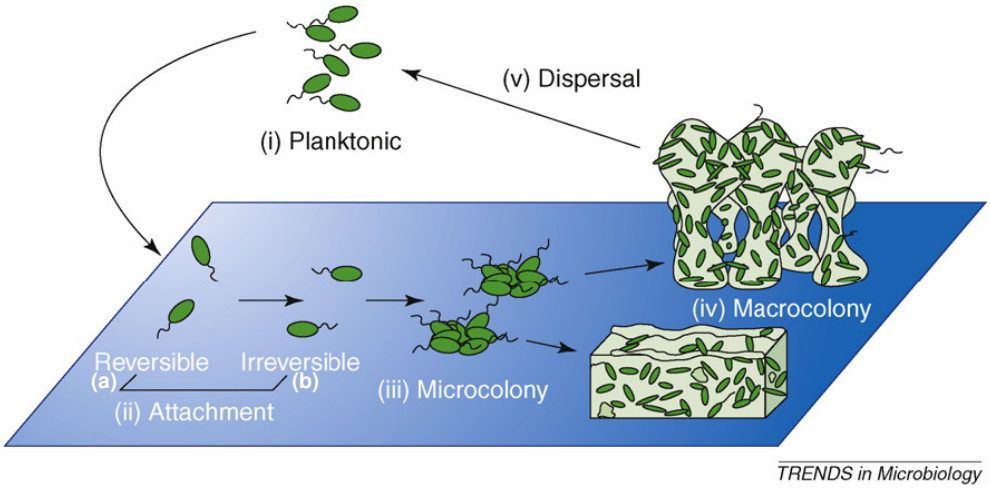

FIG. 1.1. Diagram of biofilm growth portraying planktonic cells (i) initiating attachment (ii), which involves reversible (a) and irreversible (b) attachment, with subsequent microcolony formation (iii), biofilm maturation or macrocolony formation (iv), and cell dispersal (v) (Monds and O'Toole, 2009).

activity therein. The term *biofilm system* on the other hand comprises the components affecting the rate of biofilm formation, biofilm structure and biofilm activity, which includes a group of four factors: the biofilm itself; the substrate surface; the surrounding solution of nutrients; and the gas phase, if present (Lewandowski and Beyenal, 2007).

Many cellular components, including proteins, polysaccharides, and nucleic acid monomers function in biofilm processes (Hall-Stoodley and Stoodley, 2002). These components form part of and influence factors, such as motility, cellular transport, hydrophobicity, EPS production and autoaggregation, which play an integral role in biofilm formation (Hall-Stoodley and Stoodley, 2002). Typical examples of biofilm processes include cell attachment, detachment and biofilm growth (Lewandowski and Beyenal, 2007).

1.1.1. Reversable Attachment

Initial attachment of bacterial cells (Fig. 1.1 ii-a), which is reversible, is largely influenced by cell surface components, such as flagella, pili, fimbriae, as well as protein and polysaccharide structures, either present on the cell surface as part

of the outer membrane, capsular material or excreted by cells; and the substratum surface properties (Donlan, 2002; Hall-Stoodley and Stoodley, 2002). Cell surface components affect the physicochemical properties of the cell surface which determine the rate and extent of attachment to a particular substratum surface (Donlan, 2002). Many of these structures, including flagella and pili, are involved in bacterial motility and a loss in bacterial motility is often accompanied by a loss in the ability to form a biofilm (Donlan, 2002; Hall-Stoodley and Stoodley, 2002).

1.1.1.1. Bacterial motility structures

Motility is believed to enhance initial interaction between the bacterial cell and a substratum by overcoming long-range repulsive forces that may hinder close association of the cell with the surface (Karatan and Watnick, 2009). The most thoroughly studied class of bacterial adhesins includes the motility structures flagella and pili (Karatan and Watnick, 2009). Flagellar motility is known to play an important role in the initial attachment of bacterial cells to a substratum (Donlan, 2002). Kirov *et al.* (2004) observed that both polar and lateral flagella of *Aeromonas* spp. play an important function in bacterial adherence and consequent biofilm formation. Flagellar mutants lacking both polar and lateral flagella showed a complete loss in the ability to form biofilms. Similarly, *Escherichia coli* biofilm formation is impeded by a lack in flagella or dysfunctional flagella (Van Houdt and Michiels, 2005).

Similar to flagella, pili are long surface appendages involved in movement and adherence of bacterial cells, but are mainly located at the poles of cells (Karatan and Watnick, 2009). The retractable ability of many types of pili is believed to play an important role in overcoming antagonistic forces that may hinder attachment (Karatan and Watnick, 2009). Type IV pili of *P. aeruginosa*, necessary for colonization of host tissue, are also involved in twitching motility and biofilm formation (Van Schaik *et al*., 2005). The *Vibrio vulnificus* type IV pilin

sharing homology with group A type pilins expressed by *V. cholerae*, *P. aeruginosa* and *A. hydrophila*, respectively, plays an important role in biofilm formation and adherence to epithelial cells (Paranjpye and Strom, 2005).

For the gliding bacteria, motility is operated by unique systems for which the mechanisms responsible for movement are still largely unknown, but does not require flagella (McBride, 2004). However, as with flagellar and twitching motility, gliding motility is important in biofilm formation and virulence (Mignot *et al*., 2007). *Myxococcus xanthus* has two gliding motility systems, viz., S motility, which is related to twitching motility and involves type IV pilus retraction; and A motility, of which the mechanism is largely unknown. Focal adhesion sites at cell poles have been demonstrated to play an important role in A motility, but it is unclear whether the motility force is generated at these sites (Mignot *et al*., 2007).

Multiple gliding mechanisms may power gliding motility observed in cyanobacteria (McBride, 2001). A gliding mechanism similar to *M. xanthus* S motility, involving the attachment and retraction of pili, has been observed in certain marine cyanobacteria. However, some filamentous cyanobacteria lack pili, and propulsion is largely associated with the excretion of polysaccharide slime (McBride, 2001), which may also aid in bacterial attachment and biofilm formation (Donlan, 2002; Hall-Stoodley and Stoodley, 2002).

Members of the phylum Bacteroidetes (*Cytophaga-Flavobacterium-Bacteroides*), on the other hand, exhibit a unique type of gliding motility. *Flavobacterium* spp. gliding motility takes place on solid surfaces, a trend observed for most gliding bacteria where movement is associated with the excretion of polysaccharides, type IV pili retraction and/or movement of the cytoskeleton (Godchaux *et al*., 1991; McBride, 2004; Mignot *et al*., 2007). However, *Flavobacterium* spp. lack pili and the mechanism of cell movement is still unclear. Using cryo-electron tomography, Liu *et al.* (2007) observed that motile *F. johnsoniae* isolates have thin filaments on the cell surface. A non-motile mutant strain, lacking the *gldF* gene necessary for gliding, did not display these cell surface filaments and was unable to bind and propel latex spheres

along the cell surface as opposed to wild type cells. These filaments have thus been suggested to mediate gliding motility and act as adhesins for the surface association of *F. johnsoniae* to a substratum, however, their function in bacterial adherence and initial attachment requires further investigation.

1.1.1.2. Other cell-surface components

Two other major classes of protein adhesins exist, viz., fimbrial adhesins, which include fimbriae and curli; and non-fimbrial adhesins, which consist of single proteins or homotrimers (Gerlach and Hensel, 2007). The expression of several adhesins of different sub-classes on the cell surface of bacteria may facilitate adaptation to different environmental conditions, and/or recognition of diverse substrates or host tissues, in the case of pathogenic organisms, during the attachment process (Gerlach and Hensel, 2007).

Proteinaceous filamentous surface appendages that facilitate bacterial attachment, but do not aid in motility, include fimbriae and curli (Van Houdt and Michiels, 2005). Fimbriae are the most common adhesins found in the family *Enterobacteriaceae* and are well known to be involved in bacterial attachment and biofilm formation (Donlan, 2002; Van Houdt and Michiels, 2005). Curli play a role in the pathogenicity of *E. coli* and also influence adherence in certain pathogenic strains (Van Houdt and Michiels, 2005). Mutations in the genes coding for curli expression in an *E. coli* K-12 strain resulted in a loss of adherence ability (Vidal *et al.*, 1998).

Non-fimbrial adhesins, including cell surface proteins, may also play an important role in initial attachment and subsequent biofilm formation. Transporter proteins and porins are important adhesins that contribute to bacterial attachment to substratum surfaces and host tissue (Van Houdt and Michiels, 2005). Several autotransporter proteins, including Ag43, AIDA and TibA, in *E. coli* have been associated with adhesive phenotypes of this organism (Van Houdt and Michiels, 2005). The porins OmpR in *E. coli* and OprF in *P. aeruginosa*, play a role in cell

adherence to both abiotic and biotic surfaces (Otto *et al*., 2001; Seyer *et al*., 2005).

Cell surface proteins other than transporters and porins may also function as adhesins during initial attachment. The BAP family proteins constitute a large group of adhesins which mediate adhesion in a number of Gram-positive and Gram-negative bacteria and also play a role in the biofilm formation process (Gerlach and Hensel, 2007). In *Staphylococcus aureus*, the prototypical cell wall protein, Bap, was demonstrated to influence initial attachment as well as autoaggregation of bacterial cells (Cucarella *et al*., 2001). In *P. aeruginosa*, mutation in the *lap* genes, which encode a series of membrane-associated proteins, including the BAP family protein LapA, resulted in impaired adhesion to quartz sand (Hinsa *et al*., 2003).

Bacterial capsules are important virulence factors in many pathogens, but capsule are also associated with biofilm formation (Norton *et al*., 2008). Bacterial capsule and proteinaceous capsular components, such as lectins, may aid in the attachment process, with subsequent colonization of surfaces (Crump *et al*., 2001; Norton *et al*., 2008). Decostere *et al*. (1999a) demonstrated that a cell surface lectin, defined as a glycoprotein, present in the capsule of a high virulent *F. columnare* strain was responsible for bacterial attachment to the gill tissue of carp. A decrease in adherence was observed in less virulent strains of *F. columnare* and transmission electron microscopy revealed less virulent strains had a much thinner capsule compared to highly virulent strains. Similarly, a sialic acid-binding lectin, which may be present in capsular material, was shown to be important in the adherence of *F. psychrophilum* (Møller *et al*., 2003). Lectins are also often present on the tips of pili, which play an important role in attachment, but pili have not yet been observed on the cell surface of *F. columnare* or *F. psychrophilum* (Møller *et al*., 2005). The *F. johnsoniae* cell surface filaments have been suggested to act like adhesins, but their physiological nature is unknown (Liu *et al*., 2007). However, lectins and capsule may also hinder the initial attachment process by masking adhesins required for bacterial attachment (Donlan, 2002). In *V. vulnificus*, capsular polysaccharide seems to inhibit

attachment and biofilm formation (Joseph and Wright, 2004). Hence, the role of these components in adherence and biofilm formation seems species-dependent (Donlan, 2002).

In addition to protein structures which aid in bacterial attachment, lipopolysaccharide (LPS) components and other polysaccharides present on or excreted on the cell surface may also aid in initial attachment (Donlan, 2002; Hall-Stoodley and Stoodley, 2002). In *S. epidermidis*, the polysaccharide intercellular adhesin/hemagglutinin was demonstrated to play an important role in bacterial adherence (Rupp *et al.*, 1999). Furthermore, the PGA polysaccharide required for adhesion in staphylococci was also shown to play an important role in attachment of *E. coli* cells to abiotic surfaces (Van Houdt and Michiels, 2005; Wang *et al.*, 2004b). Tsuneda *et al.* (2003) demonstrated that hexose and pentose polysaccharide constituents of cell-surface EPS promoted bacterial adherence to glass surfaces and that cell adhesion was enhanced by polymeric interaction.

The interaction and presence or absence of cell surface macromolecules, such as fimbriae, curli, outer membrane proteins (OMPs), mycolic acids, lipopolysaccharide (LPS) and other polysaccharide constituents, determine the physicochemical nature of a particular bacterial cell surface (Bendinger *et al.*, 1993; Donlan, 2002). The physicochemical properties which are determined by the chemical composition of the cell wall affect not only initial attachment, but may also influence the rate and extent of bacterial attachment to a particular substratum (Donlan, 2002).

1.1.1.3. Physicochemical properties and bacterial attachment

It is believed that the negative electrokinetic potential of the cell surface and substratum regulates bacterial adhesion (Bendinger *et al.*, 1993; and Van Loosdrecht *et al.*, 1987). Therefore, the molecular mechanism of bacterial attachment requires an understanding of physicochemical properties and

cellular-substratum interaction. Besides the physiological state of bacterial cells, physicochemical properties of the cell surface are believed to play an integral role and probably the most important role in bacterial adhesion and biofilm formation (Donlan, 2002). These properties involve intercellular and cellular to substratum adhesion interactions, respectively, arising from forces, i.e. surface charge and polarity, present on both the cell surface and the surface of the substratum.

Hydrophobic interactions tend to increase with increasing non-polar forces between two surfaces, which decrease repulsive electrostatic interaction (Donlan, 2002). Hence, generally microorganisms with more hydrophobic surfaces attach more readily and faster to surfaces of hydrophobic nature (non-polar) such as PVC, Teflon and other plastics (Donlan, 2002; O'Toole and Kolter, 1998b). Although a number of exceptions are being reported (Basson *et al*., 2008; Coquet *et al*., 2002a).

Cell surface structures that contribute to surface hydrophobicity include fimbriae, OMPs and mycolic acids (Donlan, 2002). Many bacteria have a net negative charge on the cell surface and/or are very hydrophilic, but still carry hydrophobic surface components, such as fimbriae, which may facilitate attachment to hydrophobic surfaces (Rosenberg and Kjellerberg, 1986). Fimbriae, for instance, often contain hydrophobic amino acid residues which contribute to the hydrophobicity of the cell surface and play an important role in bacterial attachment (Donlan, 2002; Rosenberg and Kjellerberg, 1986). Coquet *et al.* (2002a) observed that *Y. ruckeri* cells were hydrophilic, yet they attached well to hydrophobic surfaces, such as PVC. Similarly, Basson *et al*. (2008) reported cells of *F. johnsoniae*-like isolates to be hydrophilic, yet these strains formed thicker biofilms more rapidly on Perspex (hydrophobic) compared to glass (more hydrophilic) surfaces. Chae *et al*. (2005) found no correlation between *Listeria monocytogenes* hydrophobicity, measured with the bacterial adherence to hydrocarbon (BATH) test, and bacterial attachment to glass. Zhang *et al*. (2007) observed that the deletion of *ycfR*, whose product YcfR inhibits *E. coli* biofilm formation on glass wool (relatively hydrophilic), increases cell surface

hydrophobicity by affecting cell surface protein gene expression, thereby facilitating biofilm growth.

The attachment process of bacteria to surfaces is very complex with many variables affecting the result. However, bacteria seem to attach more readily to surfaces which are hydrophobic, rough in texture and covered in conditioning films (Donlan, 2002). Cell-surface polysaccharides and LPS, on the other hand, seem to be more important in attachment to hydrophilic surfaces (Donlan, 2002).

1.1.1.4. Substratum surface properties and bacterial attachment

Together with surface charge and polarity, surface roughness of solid supports is also recognized as a major contributing factor to initial cell attachment and consequent biofilm formation (Coquet *et al.*, 2002a; Donlan, 2002). Shear forces of the flowing medium are reduced as the surface roughness of the substratum increases (Donlan, 2002). Additionally, the surface area on a rough surface is greater and bacterial attachment and colonization of surfaces seems to increase with increased surface roughness (Donlan, 2002). Apart from substratum surface properties, conditioning films, consisting of a diversity of macromolecules and smaller molecules adsorbed to a surface, may also form and alter the properties of the original surface, greatly affecting bacterial attachment. A conditioning film may alter the physicochemical characteristics of a surface; it can present a source of nutrients as well as required trace elements essential for microbial growth; or detoxify the surface, such as in the case of metals releasing toxic ions, or the surrounding environment by binding inhibitory molecules, providing a favourable surface-environment for bacterial attachment (Lewandowski and Beyenal, 2007).

1.1.2. Irreversible Attachment

Once initial attachment has taken place, irreversible attachment (Fig. 1.1 ii-b) is manifested with the production of EPS necessary for aggregation and formation of the biofilm structure (Donlan, 2002; Hall-Stoodley and Stoodley, 2002). The production of EPS is also regarded as an adaptive measure by bacteria to persist under unfavourable conditions (Donlan, 2002; Jefferson, 2004). It is functional as defense against shear forces, protection against desiccation and phagocytosis and it also confers antimicrobial resistance (Donlan, 2002; Jefferson, 2004; Sutherland, 2001; Webb *et al*., 2003).

Cell-surface components, such as ABC transporters and lipoproteins are involved in the export of EPS necessary for irreversible attachment (Haft *et al*., 2006; Norton *et al*., 2008; Silver *et al*., 2001). An ABC transporter was demonstrated to play a role in the transition between reversible and irreversible attachment of *P. fluorescens* through the excretion of the large membrane bound protein, LapA (Hinsa *et al*., 2003). Proteins that belong to the PEP-CTERM protein family, which include lipoproteins, are also associated with EPS production in Gram-negative soil bacteria known to form biofilms (Haft *et al*., 2006; Norton *et al*., 2008).

Irreversible attachment with the production of EPS is followed by cell division and microbial growth. This leads to the formation of small isolated colonies, a process termed microcolony formation (Hall-Stoodley and Stoodley, 2002).

1.1.3. Microcolony Formation and Biofilm Maturation

Division of attached bacterial cells produces discrete multicellular aggregates, known as microcolonies (Fig. 1.1 iii), which are randomly scattered across the surface of the substrate (Webb *et al*., 2003). Besides binary division of attached cells, autoaggregation may also play an important role in microcolony formation

(Hall-Stoodley and Stoodley, 2002; Van Houdt and Michiels, 2005). Surface-associated aggregation, i.e. autoaggregation and co-aggregation, of bacterial cells can recruit cells from the bulk fluid of the surrounding environment (Hall-Stoodley and Stoodley, 2002).

Cell-surface components often determine aggregation of bacterial cells (Kolenbrander *et al*., 2000). Autoaggregation of *E. coli* cells is established through Ag43-Ag43 interactions and microcolony formation is induced by this autotransporter protein (Ag43) (Danese *et al*., 2000; Van Houdt and Michiels, 2005). The PGA polysaccharide produced by *E. coli* has also been demonstrated to be involved in intercellular adhesion and subsequent biofilm formation (Wang *et al*., 2004b). Similarly, the polysaccharide intercellular adhesin, PIA, mediates microcolony formation in *S. aureus* and *S. epidermidis* (Stanley and Lazazzera, 2004). As the microcolonies develop, they fuse to form a three dimensional biofilm structure (Fig. 1.1 iv) containing a complex layered bacterial community representing multicellular differentiation.

Complex cell signaling processes seem to be involved in the differentiation of bacterial cells during the development of the mature biofilm. This communication between bacteria, termed quorum sensing, is achieved through the production of small cell signaling molecules, generally acylated homoserine lactones (AHLs) in Gram-negative bacteria and peptides in Gram-positive bacteria (Jefferson, 2004; Stickler, 1999). At a certain population threshold density, accumulating cell signaling molecules may reach a critical concentration which affects gene expression (Jefferson, 2004; Stickler, 1999). Although the primary function of the cell signaling process remains unclear, cell signaling molecules may readily bind to cell surface receptors facilitating the expression of certain genes involved in the development of the mature biofilm cellular structure (Jefferson, 2004; Stickler, 1999).

Cell signaling is also thought to play a role during initiation of the biofilm mode of growth, however, contradictory results have made this hypothesis unclear. No cell signaling molecules were present during initial attachment of *Pseudomonas putida* and Sauer and Camper (2001) suggested that quorum

sensing did not play a role in the changes observed in protein patterns and gene expression of this organism during early biofilm development. Las quorum sensing of *P. aeruginosa* was shown to play an important role during the later stages of biofilm development (Sauer *et al*., 2002). Activation of the *las* regulon manifested as irreversible attachment and initiated cell cluster formation under continuous flow conditions (Sauer *et al*., 2002). Similarly, Davies *et al*. (1998) observed that cell signaling systems of *P. aeruginosa* were involved in the biofilm differentiation process rather than initial attachment and play an important role in biofilm maturation.

Once the mature biofilm is established, cells and cell aggregates are continuously released from the surface layer of the biofilm (consisting of actively dividing cells) into the surrounding medium (Fig. 1.1 v). Sauer *et al*. (2002) demonstrated that *P. aeruginosa* cells within cell clusters actively swim away from the interior of these clusters formed during biofilm maturation, a process referred to as dispersion. After leaving the biofilm, shell-like structures with walls of non-motile bacteria were left behind, possibly facilitating better nutrient access (Sauer *et al*., 2002).

1.2. THE BIOFILM PHENOTYPE

Most of the current knowledge on bacterial behaviour is based on free-living bacteria, which constitute only a small percentage of natural bacterial growth (Davies, 2000), and would not be relevant to surface-associated bacteria. Bacterial cells that grow in the biofilm mode are phenotypically different from their planktonic counterparts (Stickler, 1999) and an increasing number of studies indicate that genetic regulation of bacteria growing in the biofilm state is distinct from planktonic bacteria (Davies, 2000; Jefferson, 2004; Karatan and Watnick, 2009; Prigent-Combaret *et al*., 1999). Hence, specific growth-related, planktonic and sessile phenotypes exist.

Global differential gene expression in biofilms varies greatly between Gram-negative and Gram-positive bacteria and between different bacterial species (Beloin and Ghigo, 2005; Sauer, 2003). Biofilm investigations have even demonstrated differences in biofilm gene expression in isolates from the same bacterial species grown under similar experimental conditions (Ghigo, 2003). Hence, biofilm growth is not simply an adaptive response to specific environmental conditions, but involves alteration in a large portion of the bacterial transcriptome, affecting major specific genetic and physiological pathways (Ghigo, 2003).

Multiple phenotypes may exist within the biofilm growth phase, which may represent structural- and physiological-related heterogeneity (Ghigo, 2003). Differences in nutrient availability, oxygen, pH and osmolarity may create physicochemical gradients in the biofilm architecture, influencing bacterial gene expression at the different levels (Ghigo, 2003). Many of the genes involved in the different biofilm processes may be replaced by others with similar function, depending on the substratum type, nutrient availability and other growth conditions (Beloin and Ghigo, 2005). Beloin and Ghigo (2005) even proposed that each biofilm might be a world of its own. Hence, no single example for the biofilm phenotype exists.

Jouenne *et al*. (2004) proposed that differentially expressed genes characteristic of biofilm colonies represent three major categories, viz., membrane, metabolic and adaptation. Biofilm studies frequently classify genes and gene products under these three major categories (Sauer and Camper, 2001, Sauer *et al*., 2002; Seyer *et al*., 2005), with the addition of transcriptional regulators (Helloin *et al*., 2003; Shemesh *et al*., 2007) and cell signaling mechanisms (Prigent-Combaret *et al*., 1999; Sauer *et al*., 2002; Stanley and Lazazzera, 2004) responsible for regulation of global gene expression.

Altered gene expression of membrane-associated components includes transporter proteins, lipoproteins, motility structures and membrane-associated chaperone proteins (Helloin *et al*., 2003; Sauer and Camper, 2001, Sauer *et al*., 2002; Seyer *et al*., 2005). The metabolic shift in biofilm cells often include

enzymes involved in amino acid metabolism, carbon flux and polysaccharide biosynthesis/degradation (Ghigo, 2003; Helloin *et al*., 2003; Sauer *et al*., 2002; Seyer *et al*., 2005; Whiteley *et al*., 2001). The adaptation process on the other hand represents genes involved in stress response, such as peroxidases and factors that remove oxygen radicals, proteins involved in intracellular glutamate regulation as part of osmoregulation, heat shock proteins and proteins involved in the evacuation of toxic substances (Helloin *et al*., 2003; Hentzer *et al*., 2005; Prigent-Combaret *et al*., 1999; Saum and Müller, 2007; White-Ziegler *et al*., 2008; Zhang *et al*., 2007). A similar trend in adaptation/stress response in biofilms has been observed in stationary phase planktonic cells (Blokpoel *et al*., 2005; Dukan and Nyström, 1998; Helloin *et al*., 2003; Kikuchi *et al*., 2005). However, although stationary phase planktonic cultures may represent a heterogeneous group of bacterial cells in different growth stages, the adaptation/stress response phenotype in biofilm cells remains unique (Beloin and Ghigo, 2005; Hentzer *et al*., 2005; Schembri *et al*. 2003; Seyer *et al*., 2005; Spoering and Lewis, 2001; Vilain and Brözel, 2006; Waite *et al*., 2005).

There is evidence of activation and de-activation, and up- and down-regulation of gene clusters in the different biofilm processes, even as early as in the initial attachment process when bacterial cells come into contact with a suitable substratum (Davies, 2000). Prigent-Combaret *et al*. (1999) observed that a large change in genetic regulation took place in *E. coli* following attachment to a solid surface, altering transcription of 38% of its genes. This differential gene expression included the activation of cell-to-cell signaling mechanisms as well as changes in gene expression regulating micro-environmental conditions of osmolarity and oxygen concentration correlated with biofilm growth (Prigent-Combaret *et al*., 1999).

It has been proposed that the dynamic physicochemical nature of the environment may modulate gene expression through complex cellular regulatory pathways (Prigent-Combaret *et al*., 1999; Stanley and Lazazzera, 2004). Environmental stimuli, especially those related to stress response in bacteria, such as nutrient stress, predation, or exposure to harmful components or other

harmful conditions, may induce microcolony formation, with or without subsequent biofilm formation, and may evoke changes in the mature biofilm to ensure optimal nutrient acquisition through complex cell-to-cell signaling mechanisms (Stanley and Lazazzera, 2004; Webb *et al*., 2003). A multiple stress resistance protein, YcfR, was found to be significantly induced in *E. coli* biofilms (Zhang *et al*., 2007). Deletion of the *ycfR* gene induced several stress response genes and also increased sensitivity to stressful conditions. Stress response genes in a *ycfR* mutant were shown to be increased three- to four-fold in the biofilm growth phase and included genes involved in acid, osmotic, oxidative, and thermal stress response as well as ethanol tolerance (Zhang *et al*. 2007).

Beyhan *et al*. (2007) showed that extracellular protein transporter genes in *V. cholerae* were positively regulated by the VpsT regulon, which is responsible for the rugose variant and biofilm formation. The rugose variant of *V. cholerae* showed a preference for the biofilm lifestyle and had an increased ability to produce an exopolysaccharide, *Vibrio* polysaccharide (VPS), required for biofilm maturation. Resistance of the rugose variant towards osmotic, acid and oxidative stress is directly related to the production of VPS. Cellular levels of c-di-GMP, which regulate *vps*, *vpsT* and *vpsR* expression, were also shown to be higher in the rugose variant (Beyhan *et al*., 2007).

A wide variety of bacterial signal transduction regulatory systems influence biofilm formation (Ghigo, 2003; Prigent-Combaret *et al*., 1999; Stanley and Lazazzera, 2004). These signal transduction regulatory systems may regulate biofilm formation at three major developmental stages, viz., bacterial attachment, microcolony formation and biofilm maturation, where the latter is further influenced in depth and architecture (Stanley and Lazazzera, 2004). Hence, complex regulatory networks form an important part of the biofilm phenotype and may further subdivide the biofilm phenotype according to the different stages that occur in biofilm development (Sauer *et al*., 2002; Stanley and Lazazzera, 2004).

Typically, two component signal transduction systems (TCSTS), and/or multi-component signal transduction systems are involved in all stages of biofilm

development (Stanley and Lazazzera, 2004). Such TCSTS, which respond to environmental changes that influence microbial growth, have been described as good candidates for the initial regulation of biofilm formation (Ghigo, 2003).

In *E. coli* attachment, there is evidence for two component regulation of the formation of stable cell-to-surface interactions through altered gene expression (Stanley and Lazazzera, 2004). Two TCRS, viz., the Cpx and EnvZ/OmpR signaling systems, have been demonstrated to stimulate stable cell-to-surface interactions in this organism (Stanley and Lazazzera, 2004). Signal transduction in the Cpx signaling system is dependent on an OMP, NlpE, which regulates the production of P-pili required for adhesion (Stanley and Lazazzera, 2004). Interestingly, the Cpx signaling system is activated when *E. coli* cells interact with a hydrophobic surface (Otto and Silhavy, 2002). The EnvZ/OmpR signaling system is activated by conditions of increasing osmolarity which induces curli production and EPS biosynthesis through regulation of the *csgAB* operon and *adrA* gene, respectively (Prigent-Combaret *et al*., 2001; Vidal *et al*., 1998).

Stable cell-to-cell interactions are required during microcolony formation (Hall-Stoodley and Stoodley, 2002). Environmental factors such as glucose, anaerobiosis, high osmolarity, high temperature, ethanol and iron limitation influences the production of PIA, required for *S. aureus* and *S. epidermidis* microcolony development, through induction of the *icaADBC* operon (Stanley and Lazazzera, 2004). High osmolarity conditions stimulate Sigma-B signal transduction which increases *icaADBC* expression, affecting microcolony formation in *S. aureus* (Stanley and Lazazzera, 2004).

The transcription factor Spo0A regulates microcolony formation in *Bacillus subtilis* (Hamon and Lazazzera, 2001). Multiple environmental and physiological stimuli regulate the production of Spo0A through a multi-component phosphorelay system. Phosphorylated Spo0A represses transcription of a microcolony repressing transcriptional factor encoded by the *abrB* gene, facilitating biofilm formation (Hamon and Lazazzera, 2001).

Once initial attachment and microcolony formation has occurred, the mature biofilm structure can be formed (Stanley and Lazazzera, 2004). Besides the physical nature of the direct environment in which the biofilm is formed, the structure of the mature biofilm may also be regulated by bacterial processes (Coquet *et al*., 2002a; Donlan, 2002; Stanley and Lazazzera, 2004). The depth of the mature biofilm may be regulated by catabolic repression (Stanley and Lazazzera, 2004). In *B*. *subtilis* and *E*. *coli*, biofilm depth or growth is either reduced or inhibited by this phenomenon, respectively (Stanley and Lazazzera, 2004). The transcriptional factor CcpA in *B*. *subtilis* reduces the depth of the mature biofilm by catabolic repression through a rapidly metabolized carbon source, such as glucose.

In *E*. *coli*, biofilm growth is regulated by several global regulatory factors which are influenced by catabolic repression through glycogen and glucose metabolism (Jackson *et al*., 2002a-b). For instance, biofilm growth is inhibited by a high glucose concentration, which affects the cAMP receptor protein (Jackson *et al*., 2002a). Similarly, the RNA-binding global regulatory protein, CsrA, which regulates carbon flux, represses biofilm formation and also activates biofilm dispersal of *E*. *coli* cells (Jackson *et al*., 2002b).

In *P. aeruginosa*, biofilm maturation has been divided into two categories, viz., maturation-1 and maturation-2 (Sauer *et al*., 2002). Significant gene up-regulation and subsequent phenotypic changes, altering cell morphology, take place during the maturation-1 phase (Sauer *et al*., 2002). As part of a possible stress response, Arc proteins, involved in anaerobic metabolism, are up-regulated in the first phase to possibly compensate with low oxygen levels present in oxygen limited areas, such as the base layer of the biofilm (Sauer *et al*., 2002). During the maturation-2 phase, biofilm mass increases to the extent where the biofilm reaches its maximum thickness. The greatest variation with respect to differentially expressed genes between the surface-attached and planktonic phases is also observed in maturation-2 (Sauer *et al*., 2002).

The depth of mature biofilms formed by *P*. *aeruginosa* may also be reduced by the transcriptional factor RpoS (Whiteley *et al*., 2001). RpoS mutants

of *P. aeruginosa* formed thicker biofilms under flowing conditions, compared to wild type cells (Whiteley *et al.*, 2001). Stress conditions, such as low nutrient conditions may induce the production of RpoS, which in this case would cause biofilm cells of *P. aeruginosa* to disperse.

Besides their role in biofilm depth of the mature biofilm, cell signaling mechanisms may also be involved in the regulation of biofilm architecture (Stanley and Lazazzera, 2004). In *B. subtilis*, surfactant production, which is regulated by quorum sensing, is required for fruiting body formation (Hamon and Lazazzera, 2001). The ComX pheromone accumulates under high cell density conditions, which activates the sensor kinase ComP required for phosphorylation of ComA, a transcription factor that regulates surfactant production (Lazazzera *et al.*, 1999). Similarly, the *lasI-lasR* quorum sensing system in *P. aeruginosa* regulates surfactant production required for the pillar and water channel structures observed in biofilms (Pearson *et al.*, 1997). This indicates that quorum sensing is required for normal biofilm architecture through regulation of surfactant production in *B. subtilis* and *P. aeruginosa*.

The complex regulatory systems involved in the different stages of and gradients within biofilms make them complex study material. Although biofilm phenotypes are distinct from that of planktonic cells, to date there is no molecular approach, including proteomic, transcriptomic or matrix analysis that specifically defines the biofilm phenotype (Karatan and Watnick, 2009).

1.3. MOLECULAR BIOFILM ANALYSIS

1.3.1. Proteomics and Two-Dimensional Gel Electrophoresis

The global investigation of gene expression at the protein level is termed proteomics, a field of study applied to the discovery of novel protein biomarkers of disease, toxicity and drug efficacy. It involves techniques that also form part of a polyphasic approach to understand fundamental mechanisms of microbial life.

Conventional proteome analysis (analysis of the proteins expressed by a genome) involves a combination of two-dimensional gel electrophoresis (2-D gel electrophoresis) and mass spectrometry (MS) (Fig. 1.2) (Graves and Haystead, 2002; Gygi *et al*., 2000).

The 2-D gel electrophoresis technique is used to separate and visualize proteins and relies on the separation of proteins according to their pI (neutral charge at a certain pH), termed isoelectric focusing (IEF), which is followed by standard one-dimensional gel electrophoresis separating proteins according to their physical properties (size and molecular weight), i.e., the two fields of separation. In the first dimension of protein separation, protein samples are dissolved in a dithiothreitol (DTT) solution, which aids in linearization of proteins and reduces the formation of sulfhydryl groups, and separated on an immobilized pH gradient (IPG) polyacrylamide gel strip in an IEF cell. Generally, a wide pH range, such as pH 3-10, is used for proper separation of sample proteins, before switching to specific pH ranges for optimizing the gel resolution. Once the protein equilibration step has been completed, normal one-dimensional polyacrylamide gel electrophoresis (PAGE) is performed to separate the proteins according to their molecular weight, representing the second plane or dimension of electrophoresis (Bai *et al*., 2006; Sanchez *et al*., 1997).

An electrophoregram displaying an array of protein spots is generated from which uniquely expressed proteins or proteins of interest can be identified. Reproducibility is important in this experiment, since a specific spot would represent a specific protein of interest and it would also determine the accuracy of the protein profile produced at the time of isolation in a specific growth phase. In order to ensure reproducibility, protein isolation is standardized and performed in triplicate. The generated 2-D electrophoresis maps are then compared graphically to ensure reproducible protein profiles. Following this, protein spots of interest are selected and excised from the gel and protein mass

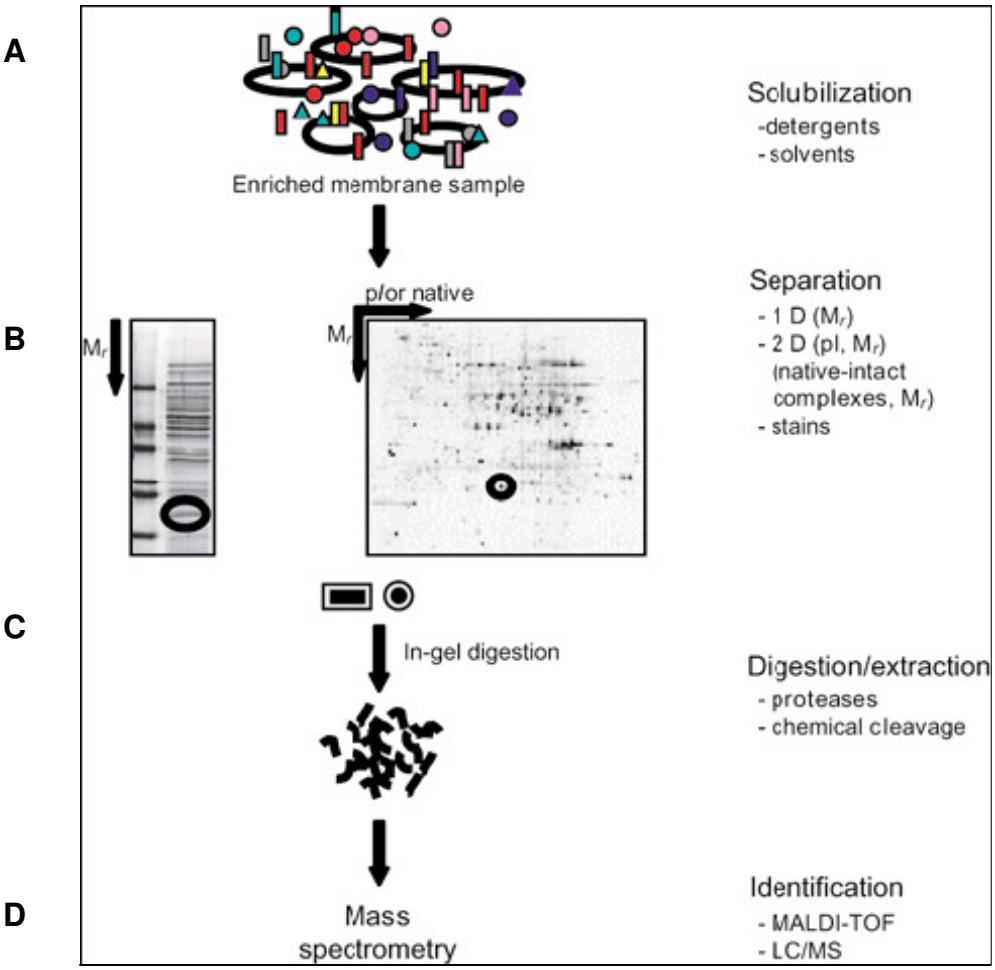

FIG. 1.2. Gel-based analysis of proteins involve four general steps: protein extracts are prepared (A) and resolved by 1-D and/or 2-D gel electrophoresis (B), selected proteins are excised from the gel and digested (C), and identified by mass spectrometry (D) (Wu and Yates, 2003).

spectra can be generated using matrix assisted laser desorption ionization - time of flight (MALDI-TOF) analysis or the amino acid sequence of selected proteins can be determined using Edman degradation (Sanches *et al.*, 1997). MALDI-TOF spectra represent peptide fingerprints which can be matched with fingerprints and/or sequences of known proteins in electronic protein sequence databases and the selected proteins are identified accordingly (Graves and Haystead, 2002; Gygi *et al.*, 2000).

The first step in the identification process of a novel protein with unknown function involves the characterization of its primary structure. This often provides sequence homology to proteins of known function, which may then reveal mutual functional properties. In combination, 2-D gel electrophoresis and mass spectrometry (MS) form a powerful, fairly reproducible and sensitive strategy for protein detection and identification (Posch *et al*., 2006b).

Proteomics investigations are challenging and factors complicating protein analysis include the number and concentration of proteins in a sample, as well as the cell state and post-translational modification of proteins (Posch *et al*., 2006b). Many techniques have been developed in the quest to identify and characterize all expressed proteins within a given cell type or growth phase (Posch *et al*., 2006a and b). While this has not been practically achieved with any technique, 2-D gel electrophoresis remains the most popular technique to analyze proteins present in a given sample. Separation of up to 5000 proteins in a single run has been achieved with 2D-electrophoresis, which is currently the only analytical technique capable of such resolving power (Posch *et al*., 2006a). For the isolation and identification of protein complexes or specific low-abundance proteins, chromatographic techniques, such as affinity chromatography, are useful, but confined to a limited number of proteins (Graves and Haystead, 2002).

A popular and very useful application of 2-D gel electrophoresis is the differential mapping of proteins present in two or more samples (Posch *et al*., 2006b), or two different growth phases of the same organism, such as the free-living and sessile (biofilm) lifestyles (Sauer and Camper, 2001). Investigations of biofilm growth and gene expression generally involve the use of 2-D gel electrophoresis for analyzing the proteome and differential expression (McLean *et al*., 2005; Sanches *et al*., 1997). Protein profiles generated using this technique assist investigators in identifying uniquely expressed, over-expressed and/or repressed proteins in a specific growth phase of interest (Sanches *et al*., 1997). Therefore, the identification of proteins by 2-D gel electrophoresis and MS

may reveal unique proteins necessary for biofilm formation in a particular microorganism.

Vilain and Brözel (2006) compared whole cell proteomes of *Bacillus cereus* with 2-D gel electrophoresis and demonstrated that the physiology of cells grown in the biofilm phase was distinct from planktonic cells in the exponential, transient and stationary phases. Sauer and Camper (2001) noted that *Pseudomonas putida* underwent a variety of metabolic changes in the early phases of biofilm formation. A proteomics approach, using 2-D gel electrophoresis, allowed the identification of differentially expressed proteins during the initial phase of *P. putida* biofilm growth. Furthermore, Sauer and Camper (2001) observed phenotypic changes in *P. putida* during the transition between planktonic and sessile growth and 2-D electrophoresis revealed fifteen proteins which were up-regulated and thirty proteins which were down-regulated in the biofilm phase of *P. putida*. Sauer *et al.* (2002) recognized five distinct stages in *P. aeruginosa* biofilm development through protein profiling with 2-D gel electrophoresis, with each biofilm stage producing a unique protein profile, different from that of planktonic cells. Changes taking place in the biofilm phase included regulation of motility, alginate production and quorum sensing (Sauer *et al*., 2002). Using 2-D gel electrophoresis, Seyer *et al*. (2005) observed a large alteration in outer membrane protein (OMP) profiles of sessile *P. aeruginosa* cells compared to planktonic cells with several isoforms of flagellin and porin proteins being present in sessile cells. In *E. coli* biofilm formation, the interaction between type 1 fimbriae and an abiotic surface may contribute to a phenotypic change observed in the OMP composition of the outer membrane (Otto *et al*., 2001).

Although the 2-D gel electrophoresis technique remains the most popular method for expression proteomics of biofilm growth, there are several drawbacks using this technique: the technique is complicated and the time needed to optimize the technique and the specialized equipment required makes it a fairly time-consuming and expensive process; proteins in a limited pI range are compared and essential biofilm proteins falling outside the selected pI range will

not be identified; and low-abundance proteins may not be detected using this technique. Hence, a polyphasic approach, using a combination of proteomics and transcriptomics, is recommended to gain a better insight in the changes taking place in gene expression during the transition between planktonic and biofilm growth (Turner *et al*. 2005).

1.3.2. Transcriptional Profiling and Related Techniques

A transcriptome represents the total set of RNA transcripts produced by ribosomal genes and other actively expressed genes in a genome at any given time (Turner *et al*., 2005). The transcriptome may vary considerably depending on the cell type and growth or developmental stage of an organism. External factors, such as environmental conditions or extracellular signals may also influence the transcriptome of microorganisms. The systematic study of the transcriptome present at a defined cellular/physiological condition is termed transcriptomics, which involves the mRNA population as the most popular study material, since it represents the total amount of RNA produced for translation (Turner *et al*. 2005). Although demanding, comparative analysis using both a transcriptomics and proteomics approach remains the most thorough study of differentially expressed genes and gene products between two populations of cells and a combination of techniques from both disciplines is also recommended for this purpose (Turner *et al*. 2005).

Northern analysis, RNase-protection assay and quantitative PCR limit investigators to only a few or a single gene. However, modern methods developed for the analysis of gene expression allow the profiling of numerous, up to thousands of genes simultaneously, as well as differentially expressed genes from different cells under distinct physiological conditions (Turner *et al*. 2005). Modern transcription profiling methods can be divided into three categories, viz., hybridization, PCR and sequencing.

A number of global genetic screens can be used to investigate biofilm gene expression. Finelli *et al*. (2003) used an *in vivo* expression technology (IVET) system to identify novel genes necessary for *P. aeruginosa* biofilm formation and development. The use of this technique (originally designed for the investigation of virulence genes of pathogenic bacteria in their host environment) in biofilm studies, however, is limited, since only uniquely expressed or up-regulated genes are detected (Finelli *et al*., 2003). Transposon-mediated mutagenesis and large-scale genetic screening is also widely used for the identification of specific genes involved in biofilm formation (Álvarez *et al*., 2006; Parkins *et al*., 2001; Prigent-Combaret *et al*., 2001; Prigent-Combaret *et al*., 1999). Although many bacterial components, including regulatory proteins, involved in biofilm formation have been identified using this method, it is extremely time-consuming and limited to a small portion of the genes of interest (Álvarez *et al*., 2006; Parkins *et al*., 2001; Prigent-Combaret *et al*., 2001; Prigent-Combaret *et al*., 1999).

To understand differential gene expression, comparative analysis of gene expression during the biofilm and planktonic growth phases to detect genes uniquely expressed, repressed or up-regulated during the biofilm phase, is of great importance (Ghigo, 2003; Miesfeld, 1999; Parkins *et al*., 2001; Sauer, 2003; Turner *et al*. 2005). Comparative analysis of differential gene expression in the biofilm and planktonic growth phases can be achieved with the use of several techniques, including microarray hybridization (Whiteley *et al*., 2001), differential-display PCR (Finelli *et al*., 2003, Miesfeld, 1999) and suppression subtractive hybridization (SSH) (Parkins *et al*., 2001).

Microarray hybridization involves the isolation of RNA from two different growth phases, viz., biofilm and planktonic phases, which is then hybridized with an array of PCR products or gene fragments, consisting of oligonucleotides or cDNA clone fragments, bound to a nylon membrane or glass slide, forming the microarray (Saccone and Pesole, 2003; Whiteley *et al*., 2001). The DNA fragments attached to the array represent sequences of known genes or partially sequenced genes with unknown function and hybridization of labeled RNA or

cDNA to the microarray reveals nucleic acid sequence homology based on fluorescence (Saccone and Pesole, 2003). This technique, however, is not very cost-effective, limiting replicate experiments.

Two basic reverse transcription PCR (RT-PCR) approaches can be used to identify differentially expressed genes, the first of which uses gel-electrophoresis to visualize randomly generated PCR products displaying differential banding patterns directly related to differences in target copy number; whereas the second is based on C_Ot hybridization kinetics and differences in primer adaptors to enhance rare PCR amplimers present at higher levels in one RT-PCR reaction compared to another (Miesfeld, 1999). False-positive results make it useful as a screening method only, and it is not recommended for generating clone libraries (Miesfeld, 1999).

Suppression subtractive hybridization (Fig. 1.3) on the other hand involves the extraction of DNA from a 'driver' strain, which is hybridized with extracted DNA from a 'tester' strain, in order to isolate sequences present in the tester strain, which are absent in the driver strain (Winstanley, 2002). Differentially expressed genes are amplified in SSH, potentially resulting in a $10^2 – 10^3$ -fold enrichment, increasing the chances for detection (Miesfeld, 1999). Although SSH can be more labour-intensive than microarrays, it is far more cost-effective making it a popular method for differential gene analysis (Miesfeld, 1999).

1.3.2.1 Suppression subtractive hybridization

SSH has been designed to identify genes present in one genome/RNA population, but absent from another (Winstanley, 2002). This technique is widely used for the identification of specific DNA molecules that distinguish closely related genomic DNA samples (Fig. 1.3) or cDNA molecules that are differentially expressed amongst two closely related mRNA populations (Rebrikov *et al.*, 2004; Winstanley, 2002). For screening differential gene expression, RNA extracted

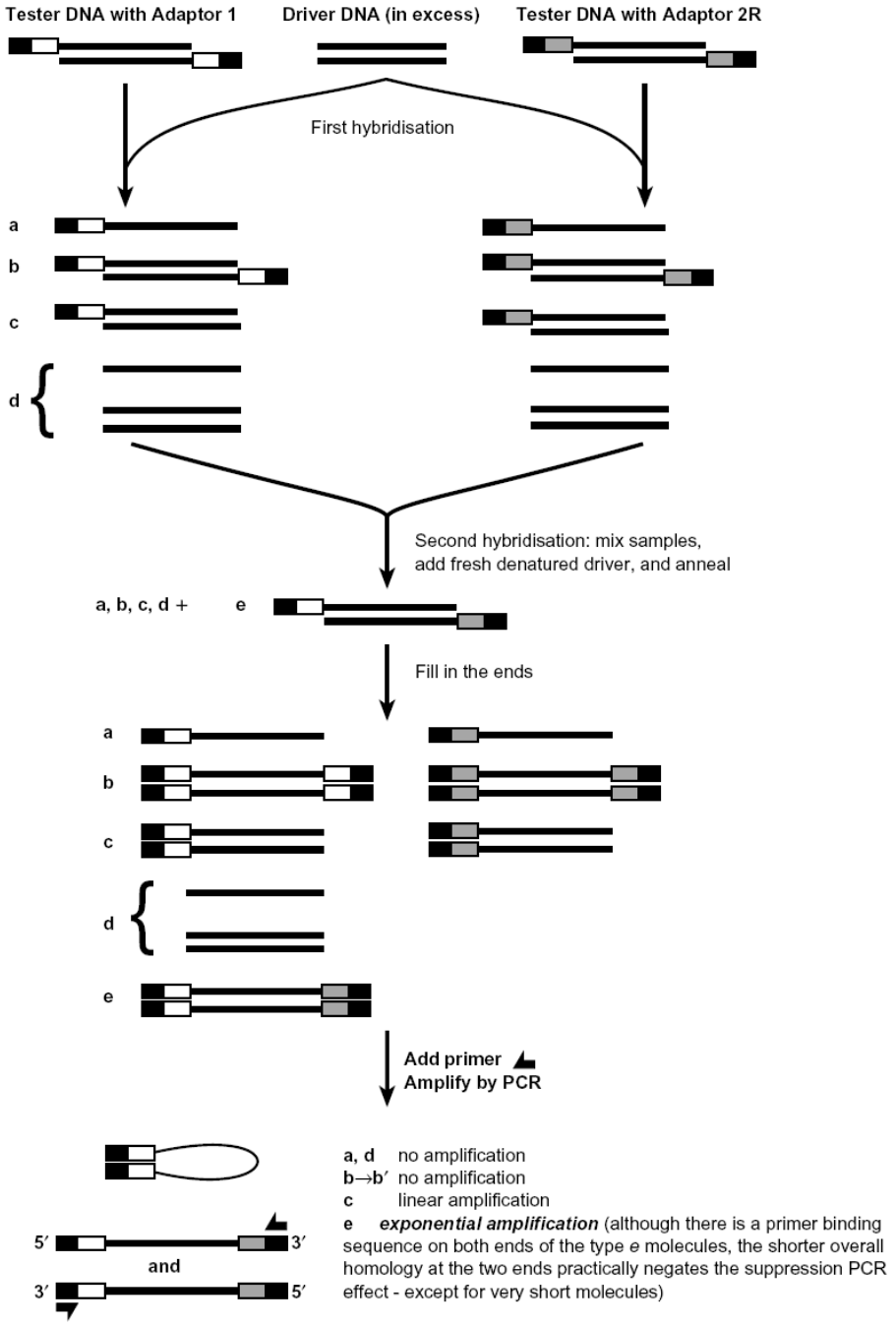

FIG. 1.3. Schematic diagram of suppression subtractive hybridization of genomic DNA (Clontech manual, Clontech, USA). “Fill in the ends” refers to filling in the missing strands of adaptor sequences by pre-incubation at 72°C in the presence of Taq polymerase and nucleotides.

from a 'driver' strain is converted to cDNA, which is hybridized with cDNA copies of RNA extracted from a 'tester' strain in order to identify the sequences that are present/up-regulated in the tester strain but absent/down-regulated and/or expressed at lower levels in the driver strain (Fig. 1.4). It is a powerful tool, making use of hybridization and PCR for generating genomic DNA or subtracted cDNA libraries and is very popular for transcriptional profiling of differentially expressed genes (Rebrikov *et al.*, 2004).

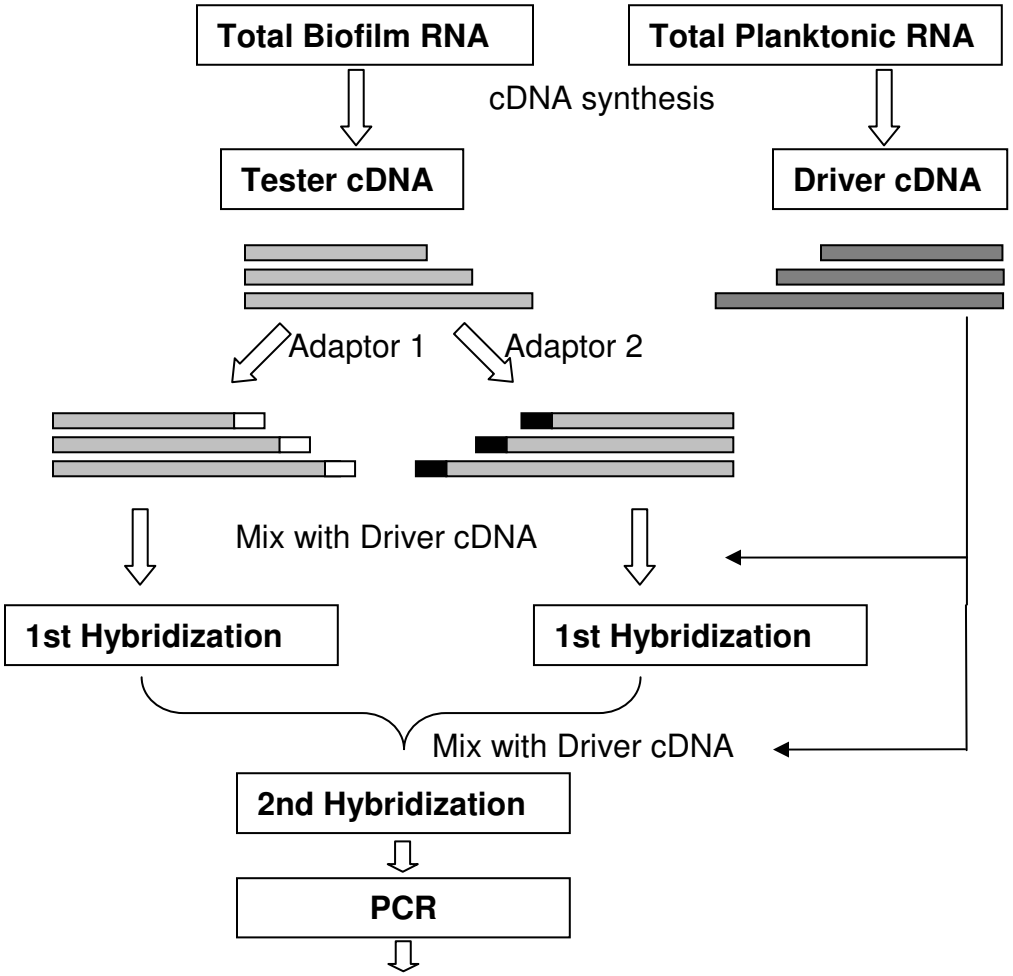

FIG. 1.4. Supression subtractive hybridization of differentially expressed biofilm genes. Total RNA from the biofilm (tester) and planktonic (driver) cells is converted to cDNA; adaptors are ligated to the biofilm-specific cDNA and subsequently hybridized twice with planktonic cDNA; PCR with adaptor-specific primers amplify cDNA gene sequences up-regulated and/or uniquely expressed in biofilm cells.

Marenda *et al*. (2005) used this technique successfully to differentiate *Mycoplasma agalactiae* from *M. bovis*, two closely related pathogenic organisms. However, this method is not only restricted to the phylogenetic study of the genomic diversity between closely related bacterial species or strains, but may readily be used to compare virulent strains of bacterial pathogens with less virulent or avirulent strains, in order to identify genomic regions present only in virulent strains (Winstanley, 2002).

Soule *et al.* (2005) focused on the genetic differences between two *F. psychrophilum* strains, one virulent and an avirulent strain, with the use of SSH. Although two distinct genetic lineages, with variation to virulence genes, were detected amongst 34 *F. psychrophilum* strains, no correlation could be established between virulence and a specific genotype (Soule *et al.*, 2005). Using SSH, Olivares-Fuster and Arias (2008) identified 110 unique *F. columnare* DNA sequences, including several coding for putative pathogenicity-related proteins and sequences related to drug resistance, when compared to DNA of a related species, *F. johnsoniae*.

Saxena *et al.* (2005) used SSH to identify unique DNA segments in virulent *Streptococcus mutans* strains associated with dental carries and suggested extension of the SSH technique should reveal unique genetic loci within a total plaque biofilm. In the study performed by Sauer and Camper (2001), a polyphasic approach using both 2-D gel electrophoresis and SSH was used to identify and compare differential gene expression between the planktonic and biofilm phases of *P. putida*. SSH revealed 40 differentially expressed genes during the initial attachment stage of *P. putida*. Comparable analysis revealed that genes involved in amino acid metabolism were down-regulated following attachment. Genes that were up-regulated in the biofilm phase included transporter proteins, such as components of ABC transporters, components of type IV pili, as well as genes involved in polysaccharide biosynthesis (Sauer and Camper, 2001).

A modified SSH technique (Parkins *et al.*, 2001), which is based on the isolation and conversion of mRNA to cDNA from both planktonic cells (driver

strain) and biofilm grown cells (tester strain), revealed actively transcribed genes unique to the biofilm phase of growth. The SSH library construction of up-regulated and/or biofilm-specific genes is a fairly new idea (Parkins *et al*., 2001) and few studies have taken this route to investigate differentially expressed genes between planktonic and surface-associated cells (Parkins *et al*., 2001; Sauer and Camper, 2001). In the case of investigating biofilm-related genes, either uniquely expressed or over-expressed, cells from the biofilm growth phase will serve as the 'tester' population and cells from the planktonic growth phase will represent the 'driver' population. Excess driver cDNA is used to facilitate the process of subtraction (Fig. 1.4). To increase the specificity of the technique, tester cDNA is digested with a restriction endonuclease, separated into two portions and separately subjected to ligation reactions to attach specific adaptor sequences to the 5' ends, respectively. The tester cDNA portions are then separately hybridized to driver cDNA and homologous sequences are mopped up, leaving behind tester-specific single-stranded sequences. Following this, the two portions are mixed, allowing homologous single-stranded cDNA to hybridize and only sequences which are unique to the tester population will have different adaptors on each strand. Primers designed to bind to tester cDNA adaptor sequences are then used to amplify these tester-specific sequences. This modified SSH technique has the potential to identify all factors that are either uniquely expressed or over-expressed in the biofilm phase of bacteria (Parkins *et al*., 2001).

1.4. THE GENUS *Flavobacterium* AND PATHOGENIC *Flavobacterium* spp.

Members of the genus *Flavobacterium* are yellow-pigmented, Gram-negative filamentous bacteria which belong to the family *Flavobacteriaceae*, one of many families constituting the phylum Bacteroidetes (Bernardet and Nagakawa, 2006). The genus *Flavobacterium* is the type genus of the family *Flavobacteriaceae*, which includes the genera *Aequorivita*, *Arenibacter*, *Bergeyella*,

Capnocytophaga, *Cellulophaga*, *Chryseobacterium*, *Coenonia*, *Croceibacter*, *Empedobacter*, *Flavobacterium*, "*Fucobacter*", *Gelidibacter*, *Mesonia*, *Muricauda*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, *Weeksella* and *Zobellia* (Bernardet and Nagakawa, 2006). These genera consist of bacterial species from widely diverse ecological niches with diverse physiological characteristics, including many pathogenic bacteria (Bernardet and Bowman, 2006).

Aquatic pathogens of the genus *Flavobacterium* infect a wide variety of farmed freshwater fish species, i.e. salmonid species, ayu, channel catfish, eels (*Anguilla rostrata*, *A. japonica*, *A. anguilla*), goldfish, tilapia (*Oreochromis* spp.), sheatfish, silver carp and common carp (Bader *et al*., 2003; Bernardet and Bowman, 2006; Wakabayashi, 1993). *Flavobacterium branchiophilum*, *F. columnare* and *F. psychrophilum* are obligate fish pathogens, which can cause mortality rates of well over 50% in farmed fish (Bernardet and Bowman, 2006). These organisms are responsible for BGD, BCD and RTFS, and columnaris disease, respectively (Bernardet and Bowman, 2006; and Wakabayashi, 1993). Fin rot prevalently observed in trout and other salmonids are also associated with *Flavobacterium* spp., especially *F. columnare* and *F. johnsoniae* (Flemming *et al*., 2007; and Wakabayashi, 1993).

Although *F. johnsoniae* has previously been described as an opportunistic pathogen (Bernardet and Bowman, 2006), *F. johnsoniae*-like isolates have been shown to play a major role in bacterial fish disease of farmed fish, especially trout, in South Africa (Flemming *et al*., 2007). *F. johnsoniae*-like isolates have been isolated from a number of commercially important fish species, viz., rainbow trout (*Oncorhynchus mykiss*), koi, Mozambique tilapia (*Oreochromis mossambicus*), sharptooth catfish and longfin eel (*Anguilla mossambica*), in South Africa (Flemming *et al*., 2007). These organisms have been isolated from external lesions and infected gills in a variety of diseased fish, of which the symptoms are very similar, often identical, to superficial lesions and gill necrosis caused by *F. columnare* (Bernardet and Bowman, 2006; Bernardet *et al*., 1996;

Darwish *et al*., 2004; Flemming *et al*., 2007). As with columnaris disease, septicemic infection and infection of the internal organs of fish occur at a later stage during the infection process, as infection progresses (Flemming *et al*., 2007). General sites of isolation include the skin, fins and gills of infected fish, however, *F. johnsoniae*-like cultures have also been isolated from internal organs of severely infected fish (Flemming *et al*., 2007). These organisms are also capable of producing biofilm structures on various surfaces present in aquaculture tanks (Basson *et al*., 2008), which may facilitate their existence and survival in the aquaculture system and may lead to recurrent disease outbreaks (Coquet *et al*., 2002b; Flemming *et al*., 2007). Besides the complications caused by persistence of pathogenic *Flavobacterium* spp. in aquaculture, members of this genus have also been associated with paper spoilage and machine system problems in the paper industry, due to the slimy exudate produced by biofilm cultures (Oppong *et al*., 2003).

Thus far, the biofilm-forming ability of *Flavobacterium* spp. and *F. johnsoniae*-like isolates in particular, has not received much attention and biofilm formation in this genus is poorly understood. The role of the gliding motility structures of *F. johnsoniae* in biofilm formation is also not clear. For instance, the role of the cell surface filaments of *F. johnsoniae* in bacterial attachment and biofilm formation has not yet been demonstrated. According to Álvarez *et al.* (2006), gliding ability and biofilm formation seem to be antagonistic properties in *F. psychrophilum*. Inactivation of the putative thiol:disulfide oxidoreductase TlpB was demonstrated to enhance biofilm growth in mutant *F. psychrophilum* cells, but abolished gliding motility (Álvarez *et al*., 2006). *F. johnsoniae*-like isolates also displayed a strong, statistically significant negative correlation between motility and the ability to adhere using a microtiter plate adherence assay (Basson *et al*., 2008). This may well indicate that the gliding machinery of *F. johnsoniae*-like isolates is not critical in primary attachment and its exact role in biofilm formation requires further investigation. Other characteristic components involved in attachment and biofilm development, such as OMPs, transporter

proteins, cell signal transduction regulatory systems and EPS biogenesis have not yet been identified in *F. johnsoniae*-like isolates.

Currently, no information is available on the proteome or transcriptome representing the biofilm phenotype of *F. johnsoniae*. Proteomic investigations, using 2-D gel electrophoresis, previously focused on the sarcosine-insoluble OMP fraction of *F. columnare* (Liu *et al*., 2008) and comparison of proteins isolated from virulent and avirulent *F. psychrophilum* strains (http://www.bio-rad.com/LifeScience/pdf/Bulletin_2670.pdf), and identification of possible antigenic targets for vaccine development (Dumetz *et al*., 2008). SSH on the other hand has been used to investigate the genomic variation between *F. columnare* and *F. johnsoniae* (Olivares-Fuster and Arias, 2008), and two *F. psychrophilum* strains (Soule *et al*., 2005), respectively. Therefore, a polyphasic study involving both techniques is required to investigate differential gene expression between the planktonic and biofilm phases of *F. johnsoniae*-like isolates.

1.5. SCOPE OF THE PRESENT STUDY

Flavobacterium spp., such as *F. columnare*, *F. psychrophilum*, *F. branchiophilum* and *F. johnsoniae*, are important fish pathogens causing high mortality in aquaculture farming worldwide. Apart from being part of the natural flora of many salmonids and difficult to isolate from their host environment (Bernardet and Bowman, 2006; Crump *et al*., 2001; Inglis and Hendrie, 1993), these organisms are capable of forming biofilms in aquaculture tanks (Basson *et al*., 2008) further complicating the control and treatment of flavobacterial disease outbreaks.

Very little is known about *Flavobacterium* spp. biofilm formation and specifically the genes involved in flavobacterial biofilm formation have not been thoroughly investigated. In order to address this problem, the present study focused on the characterization and identification of differentially expressed genes and proteins involved in *F. johnsoniae*-like isolate biofilm formation using

SSH and two-dimensional gel electrophoresis, respectively. This polyphasic approach also allowed the investigation of the relationship between phenotypic changes and genetic expression in biofilm formation.

The molecular study of differentially expressed genes and proteins involved in biofilm formation could lead to a breakthrough in understanding biofilm formation of pathogenic *Flavobacterium* spp., and many other bacterial pathogens, and the identification of novel genes and/or proteins involved in biofilm formation and virulence of *Flavobacterium* spp.

1.5.1. Hypotheses to be Tested

It is hypothesized that phenotypic and genotypic characterization of *F. johnsoniae*-like isolates will identify measurable associations between planktonic- and biofilm-grown cells. It is also hypothesized that 2-D gel electrophoresis and sequencing of proteins from biofilm and planktonic growth phases of *F. johnsoniae*-like isolates will allow identification of differentially expressed proteins involved in biofilm formation. It is further hypothesized that the comparative analysis of reciprocal suppression subtractive hybridization libraries will facilitate the assessment of differential gene expression of planktonic and biofilm-associated *F. johnsoniae*-like isolates.

1.5.2. Objectives

The following objectives have been established:

a. To investigate the association between phenotype and/or genotype of *F. johnsoniae*-like isolates with biofilm growth;
b. To identify differential protein expression between planktonic, biofilm and surface-associated *F. johnsoniae*-like isolates by 2-D gel electrophoresis;

c. To investigate differential gene expression between planktonic and biofilm-associated *F. johnsoniae*-like isolates by suppression subtractive hybridization; and
d. To identify the biofilm phenotype expressed by *F. johnsoniae*-like isolates using comparative transcriptome and proteome analysis.

1.5.3. Experimental Design

The following aims were pursued:

Chapter 2:

a. To characterize *F. johnsoniae*-like isolates by pulsed-field gel electrophoresis (PFGE);
b. To investigate the chitin-degrading ability of the *F. johnsoniae*-like isolates by a agar plate chitinase assay; and
c. To investigate EPS production of the *F. johnsoniae*-like isolates by extra-cellular carbohydrate content (ECC) analysis.

Chapter 3:

a. To identify differences in outer membrane (OMP, LPS) profiles of planktonic, biofilm and surface-associated cells by 1-D SDS-PAGE; and
b. To investigate differential protein expression between planktonic, biofilm and surface-associated *F. johnsoniae*-like isolates by 2-D gel electrophoresis.

Chapter 4:

a. To investigate differential gene expression between planktonic and biofilm-associated *F. johnsoniae*-like isolates by SSH; and
b. To identify genes uniquely expressed and/or differentially-expressed in the biofilm phase of *F. johnsoniae*-like isolates by SSH.

CHAPTER TWO

CORRELATION OF BIOFILM-FORMING ABILITY WITH PHENOTYPIC (CHITIN DEGRADATION AND EXTRACELLULAR CARBOHYDRATE CONTENT) AND GENOTYPIC (PULSED-FIELD GEL ELECTROPHORESIS) CHARACTERISTICS OF *F. johnsoniae*-like ISOLATES

2.1. INTRODUCTION

Flavobacterium johnsoniae and other members of the phylum Bacteroidetes play a specialized role in the degradation and uptake of dissolved organic material, especially complex biopolymers such as cellulose and chitin, which form part of the high molecular mass fraction of dissolved organic matter in aquatic environments (Kirchman, 2002). *F. johnsoniae* typically inhabits moist soil and aquatic environments rich in organic matter and the ability of this organism to break down chitin, especially in soil, is believed to play a role in the degradation process of invertebrate carcasses (Bernardet and Bowman, 2006). *F. johnsoniae* is also frequently isolated from water samples, indicating its ability to survive in the free-living form in aquatic environments.

The survival and persistence of *Vibrio cholerae* in the natural environment is linked to its ability to adhere to and form biofilms on chitinaceous surfaces (Pruzzo *et al*., 2008; Reguera and Kolter, 2005). The attachment of *V. cholerae* cells to chitinaceous surfaces and subsequent biofilm formation is mediated through pili, such as type IV pili (Pruzzo *et al*., 2008; Reguera and Kolter, 2005). The *V. cholerae*-chitin relationship encompasses several important characteristics of this aquatic pathogen, including physiological responses such as biofilm formation, induction of competence, symbiotic relationship with higher organisms, cycling of nutrients in the aquatic environment and most importantly, pathogenicity to humans and aquatic animals (Pruzzo *et al*., 2008). It may well be that the colonization of chitinaceous surfaces and detritus-associated biofilm

communities gives rise to the abundance of *F. johnsoniae* in fresh water (Kirchman, 2002). This may serve as a possible explanation for *F. johnsoniae*-associated fish disease, since water would be mostly deprived of organic matter, compared to soil or detritus aggregates, forcing this organism to enter an attached, pathogenic lifestyle.

F. johnsoniae cells lack pili, however, surface filaments have been associated with the ability of this organism to glide on solid surfaces (Liu *et al*., 2007). Additionally, chitin degradation of *F. johnsoniae* has been linked to the ability of this organism to glide on solid surfaces. Chang *et al*. (1984) observed that non-gliding *F*. *johnsoniae* mutants lacked the ability to degrade chitin. Disruption of the *gldA*, *gldB*, *gldD*, *gldF*, *gldG* and *gldI* genes involved in *F. johnsoniae* gliding motility also disrupted its ability to degrade chitin (McBride *et al*., 2003; McBride and Braun, 2004).

The abilities of *F*. *psychrophilum* to glide and form biofilms have been found to be antagonistic properties (Álvarez *et al*., 2006). Similarly, *F*. *johnsoniae*-like isolates displaying strong gliding motility exhibit a weak biofilm phenotype when grown on abiotic surfaces (Basson *et al*., 2008). In addition to a correlation between motility and the chitin degrading ability (McBride and Braun, 2004), a significant negative correlation between biofilm formation and gliding motility of these organisms has been observed (Álvarez *et al*., 2006; Basson *et al*., 2008). However, no correlation has been established as yet between the biofilm-forming ability and chitin degradation of *F*. *johnsoniae*-like isolates and this requires further investigation.

Biofilm formation is generally associated with the production of EPS, especially during the early stages of biofilm development (Danese *et al*., 2000; Donlan, 2002; Hall-Stoodley and Stoodley, 2002; Jefferson, 2004; Stanley and Lazazzera, 2004; Wang *et al*., 2004b). EPS often constitute large amounts of extracellular carbohydrate, aiding in cellular attachment and protection of biofilm cells. Kives *et al*. (2006) found compositional differences in carbohydrate content of biofilm and planktonic *P*. *fluorescence* EPS, respectively. A combination of glucuronic and guluronic acid were the main components in biofilm EPS, besides

rhamnose, glucose and glusosamine, whereas only glucuronic acid was present as the main carbohydrate in planktonic EPS (Kives *et al*., 2006). Quantifying the amount of EPS produced by bacteria can be troublesome, not only due to the complex nature of EPS but also because of the difficulty involved in separation of true EPS and other polysaccharides such as LPS (Ryu and Beuchat, 2003). Ryu and Beuchat (2003) developed an assay to estimate the total amount of carbohydrate present in the extracellular layer of carbohydrate produced by cells grown on an agar medium. Fraction I obtained during the isolation of extracellular carbohydrate complexes (ECC) consists of slimy EPS, traces of capsular EPS and other cell surface polysaccharides, including mono- and oligosaccharides secreted by the cell. Fraction II would mainly consist of capsular EPS and cell surface-associated polysaccharides (Ryu and Beuchat, 2003).

In *V. cholerae*, *Vibrio* polysaccharide (VPS) is associated with the rugose colony phenotype and its biofilm-forming ability (Beyhan *et al*., 2007). Strains that lack VPS appear as smooth colonies and lack the ability to form complex, mature biofilms (Beyhan *et al*., 2007). A similar difference in colony morphology, i.e., hazy and smooth, has been observed amongst the *F. johnsoniae*-like isolates (Flemming *et al*., 2007). *Flavobacterium* spp. are known to produce excessive EPS during colonization of solid surfaces and have previously been associated with paper spoilage due to the production of this slimy exudate (Oppong *et al*., 2003). The differences in extracellular carbohydrate production of smooth and hazy colonial phenotypes are unknown and the role of EPS in *Flavobacterium* spp. biofilm formation is unclear.

Moreover, extracellular polysaccharides and proteins present in bacterial capsules have been suggested to act as adhesins (Decostere *et al*., 1999a and b; Kroncke *et al*., 1990). However, the function of this carbohydrate capsular material in *F. johnsoniae* adherence, virulence and biofilm formation is poorly understood. In *F. columnare*, the carbohydrate content of capsule is associated with adherence to gill tissue. High virulence strains, with increased ability to adhere to gill tissue, have a thicker capsule layer compared to low virulence

strains (Decostere *et al*., 1999a and b). As with *F. columnare*, capsule presence has also been observed for *F. johnsoniae*-like isolates (Flemming, 2006). Comparative quantification of the exopolysaccharide production of *F. johnsoniae*-like isolates grown in the planktonic and sessile state may reveal the role and relevance of EPS in *Flavobacterium* spp. biofilm formation.

Apart from their application for the rapid identification of many pathogenic bacteria from infectious disease outbreaks, molecular typing techniques are also effective for the classification of bacterial species and phylogeny, as well as for distinguishing between specific phenotypic traits among bacterial strains (Borucki *et al*., 2003; Somers *et al*., 2001). The food-borne pathogen, *Listeria monocytogenes*, has been classified into two major phylogenetic divisions, Division I and II, with the use of molecular analysis and typing techniques, including pulsed-field gel electrophoresis (PFGE) (Borucki *et al*., 2003; Brosch *et al*., 1994). Borucki *et al*. (2003) observed that *L. monocytogenes* strains from Division II displayed increased biofilm formation compared to strains from Division I. Similarly, genomic profiling with PFGE analysis verified the presence of biofilm-forming strains of nonstarter lactic acid bacteria in cheese spoilage (Somers *et al*., 2001).

Described as the gold standard of molecular typing techniques for bacterial pathogens (Barett *et al*., 2005), PFGE facilitates the typing of large groups of a broad range of bacterial species (Tenover *et al*., 1995). The restriction patterns generated by PFGE are highly specific to different strains of bacteria and its high discrimination power adds significant value to investigations of pathogenic organisms responsible for disease outbreaks (Gautom, 1997; Tenover *et al*., 1995). PFGE has been used to differentiate amongst *F. columnare* and *F. psychrophilum* isolates obtained from a diversity of diseased fish species (Arai *et al*., 2007; Chen *et al*., 2008; Soto *et al*., 2008). Following intra-species typing of *F. columnare* strains obtained from channel catfish, large-mouth bass, red pacu, carp and brown trout, PFGE was found to be a highly reproducible and powerful epidemiological tool for discriminating between *F. columnare* isolates, regardless of the fish host species (Soto *et al*., 2008). Arai *et*

al. (2007) demonstrated that PFGE typing of *F. psychrophilum* isolates, obtained from diseased ayu, using restriction endonucleases *Bln*I and *Xho*I, enabled more accurate classification of the isolates compared to conventional RFLP analysis. Although the genetic diversity among South African *F. johnsoniae*-like isolates has been previously investigated by 16S rRNA gene sequence analysis, 16S rRNA gene PCR restriction fragment length polymorphism (RFLP) analysis, randomly amplified polymorphic DNA (RAPD) PCR and repetitive extragenic palindromic (REP) PCR (Flemming *et al*., 2007), the genetic relatedness of these isolates still remains unclear.

F. johnsoniae-like isolates are known to form biofilms in aquaculture tanks (Basson *et al*., 2008). The presence of these bacteria in aquaculture and aquatic systems in the biofilm state may contribute to recurrent disease outbreaks in fish, especially trout (Basson *et al*., 2008; Flemming *et al*., 2007). Although previously described as an opportunistic fish pathogen, this organism is the leading cause of flavobacterial disease, being the primary infectious agent in trout in South Africa (Flemming *et al*., 2007). Besides the continuous release of bacterial cells from abiotic surface-associated colonies present in aquaculture settings, detritus-associated communities of microorganisms abundant in freshwater aquatic systems also continuously release bacteria into the surrounding environment (Kirchman, 2002).

In the present study, the ability of *F. johnsoniae*-like isolates to degrade chitin and the amount of ECC produced by these isolates in planktonic phase and from agar surface-associated growth was investigated. The association between these characteristics and the biofilm-forming phenotypes was assessed. Additionally, *F. johnsoniae*-like isolates were differentiated on the basis of genetic diversity by using PFGE. Since 16S rRNA gene PCR-RFLP analysis provided poor discrimination of *F. johnsoniae*-like isolates (Flemming *et al*., 2007), RFLP analysis of the entire genome using PFGE was used for detailed genotyping. The relationship between PFGE patterns and specific biofilm phenotypes was also investigated.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial Strains and Growth Conditions

Twenty-eight *F. johnsoniae*-like isolates, previously isolated from moribund and healthy fish (rainbow trout, koi, and longfin eel), were selected for study (Flemming *et al*., 2007). They have been previously characterized at the molecular level using 16S rRNA gene sequence analysis, 16S rRNA PCR-RFLP analysis, RAPD-PCR, REP-PCR, WCP and OMP profiling, respectively (Table 2.1) (Flemming *et al*., 2007). Additionally, colonial morphology, gliding motility and their ability to form biofilms and associated adherence characteristics (hydrophobicity, autoaggregation, coaggregation and Congo red uptake) have also been previously investigated (Basson *et al*., 2008; Flemming *et al*., 2007).

F. johnsoniae-like isolates were grown in enriched Anacker and Ordal's broth (EAOB) at 26 °C for planktonic growth and cultured on enriched Anacker and Ordal's agar (EAOA) for surface-associated growth at room temperature (RT; ~ 23 °C), respectively.

2.2.2. Chitin Degradation

Colloidal chitin was prepared according to a method described by Hsu and Lockwood (1975). Colloidal chitin (4 g/l) was incorporated into EAOA and 10 µl of overnight cultures (adjusted equivalent to a 0.5 McFarland standard) was spotted onto the chitin-containing agar. Plates, inoculated in triplicate, were incubated at room temperature for 15 d. Subsequent clearing of the chitin medium underlying the bacterial colonies was considered a positive result. Zone diameters were measured and the average zone size was calculated for each respective isolate over the 15 d period. When no zone formation was observed following the 15 d incubation period, *F. johnsoniae*-like isolates were classified as negative for chitin degradation (McBride *et al*., 2003; McBride and Braun, 2004).

TABLE 2.1. Fish host species and molecular characterization of *F. johnsoniae*-like isolates*

Isolate	Fish host	16S rRNA gene PCR-RFLP genomovars†			RAPD subtypes‡	REP subtypes§	WCP subtypes¶	OMP subtypes‖	PFGE types**
		*Cfo*I	*Msp*I	*Taq*I					
YO12	Rainbow trout	C-A	M-A	T-A	R1	E2	WP1	O1	NT
YO15	Rainbow trout	C-A	M-A	T-A	R1	E2	WP1	O2	NT
YO19	Rainbow trout	C-A	M-B	T-B	R2	E3	WP3	O3	PF1
YO20	Koi	C-A	M-B	T-B	R2	E4	WP3	O4	PF1
YO21	Rainbow trout	C-A	M-B	T-B	R2	E5	WP3	O4	PF1
YO26	Rainbow trout	C-A	M-B	T-B	R2	E5	WP3	O4	PF1
YO34	Rainbow trout	C-A	M-B	T-B	R2	E5	WP3	O4	PF1
YO35	Rainbow trout	C-A	M-B	T-B	R2	E5	WP3	O4	PF1
YO38	Rainbow trout	C-A	M-B	T-B	R2	E5	WP3	O4	PF1
YO45	Rainbow trout	C-A	M-B	T-A	R3	E6	WP1	O5	PF2
YO49	Rainbow trout	C-A	M-A	T-A	R4	E7	WP1	O6	PF3
YO50	Rainbow trout	C-A	M-A	T-A	R4	E7	WP1	O6	PF3
YO51	Rainbow trout	C-A	M-B	T-B	R5	E8	WP4	O7	PF4
YO52	Koi	C-A	M-B	T-B	R6	E9	WP3	O8	PF5
YO53	Koi	C-A	M-A	T-B	R1	E2	WP1	O9	PF6
YO54	Koi	C-A	M-A	T-B	R1	E2	WP1	O9	PF7
YO55	Koi	C-A	M-B	T-B	R6	E9	WP3	O10	PF8
YO56	Koi	C-A	M-B	T-B	R6	E9	WP3	O10	PF9
YO57	Longfin eel	C-A	M-B	T-B	R7	E10	WP2	011	PF10
YO59	Longfin eel	C-A	M-B	T-B	R7	E10	WP3	O12	PF11
YO60	Longfin eel	C-A	M-B	T-B	R7	E10	WP2	O11	PF12
YO61	Longfin eel	C-A	M-B	T-B	R7	E10	WP2	O13	PF13
YO62	Longfin eel	C-A	M-B	T-B	R7	E10	WP2	O11	PF14
YO63	Longfin eel	C-B	M-B	T-B	R8	E11	WP3	O14	PF15
YO64	Longfin eel	C-B	M-B	T-B	R8	E11	WP3	O14	PF15
YO65	Biofilm	C-A	M-B	T-B	R9	E12	WP4	O15	PF16
YO66	Biofilm	C-A	M-B	T-B	R10	E13	WP3	O16	PF17
YO67	Biofilm	C-A	M-B	T-B	R9	E12	WP4	O15	PF18

* (Flemming *et al*., 2007)

† C-A → B, M-A → B, and T-A → B, respectively, represent subtypes obtained following 16S rRNA gene PCR-RFLP analysis with restriction endonucleases *Cfo*I, *Msp*I, and *Taq*I, respectively.

‡ R1-R10 represent RAPD-PCR subtypes.

§ E1-E10 represent REP-PCR subtypes.

¶ WP1-WP4 represent whole cell protein profile subtypes.

‖ O1-O4 represent outer membrane protein profile subtypes.

** PF1-PF18 represent pulsed-field gel electrophoresis subtypes; *F. johnsoniae*-like isolates YO12 and YO15 were not typeable (NT).

2.2.3. Quantification of Extracellular Carbohydrate Complexes

The ECC of *F. johnsoniae*-like isolates was assessed according to the protocol described by Ryu and Beuchat (2003) with modifications. To mimic surface attachment, overnight (O/N) broth cultures (adjusted equivalent to a 0.5 McFarland standard) were swabbed onto EAOA plates. Plates were incubated at 26 °C for a 5 d period before bacterial growth was harvested and suspended in 10 ml 0.15 M NaCl. Cell suspensions were vortexed and the OD of cell suspensions were standardized at 750 nm (OD_{750nm}). Suspensions were centrifuged at 10 000 *g* for 20 min at 4 °C. Supernatants (S-1) were decanted into test tubes and kept for ECC fraction I analysis. Cell pellets were resuspended in 10 ml of 0.15 M NaCl by vortexing and incubated in a water bath at 100 °C for 10 min. After cooling on ice, cell suspensions were centrifuged at 10 000 *g* for 20 min at 4 °C. Resulting supernatants (S-2) were decanted into test tubes and kept for ECC fraction II analysis. Cell pellets were resuspended in 10 ml 0.15 M NaCl and OD readings were taken at 750 nm.

Total carbohydrate content was quantified using the phenol-sulfuric acid method described by Dubois *et al*. (1956). Briefly, 2 ml of each S-1 and S-2 fraction, respectively, was pipetted into spectrophotometric tubes and 0.05 ml of 80% phenol was added. Five milliliters of concentrated sulfuric acid was rapidly added and the tubes were left to stand at RT for 10 min. Tubes were then shaken and placed at 30 °C for 15 min. Absorbance was measured at 480 nm (for the detection of pentoses and uronic acids) and 490 nm (for the detection of hexoses), respectively. Blanks were prepared by substituting ddH_2O with the sugar solution. Standardized ECC concentrations were calculated by dividing the total ECC (sum of S-1 and S-2) by the cell turbidity (OD_{750nm}). ECC of planktonic cells was also investigated by characterization of 50 ml EAOB cultures which had been incubated at 26 °C for 5 d. Broth cultures were centrifuged at 10 000 *g* for 20 min at 4 °C and resuspended in 10 ml 0.15 M NaCl. The OD of cell suspensions were standardized at 750 nm (OD_{750nm}). All tests were performed in triplicate for each bacterial isolate tested.

2.2.4. Preparation and Cleavage of DNA in Agarose and PFGE

Genomic DNA was prepared using a modification of the technique described by Poh and Lau (1993). O/N EAOB cultures of *F. johnsoniae*-like isolates were harvested by centrifugation and washed once with cold PBS (pH 7.4). Endogenous DNase activity was inactivated using a modification of the formaldehyde treatment described by Soto *et al*. (2008). Cells were resuspended in 0.5 ml of cold PIV [10 mM Tris-HCl (pH 7.5) and 1 M NaCl] containing 6% (v/v) formaldehyde solution and incubated on ice for 1 h. Cells were then washed twice with cold PIV and resuspended in 500 µl cold PIV. Cell suspensions were then mixed with an equal volume of 2% low-melting-point agarose (Roche, Germany). Solidified plugs were incubated in 10 ml EC lysis solution (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0.5% Triton-X 100, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 10 mg lysozyme, and 10 U RNase) O/N at 37 °C. The EC lysis solution was replaced with 5 ml ESP solution [0.5 M EDTA (9.0-9.5), 1% sodium lauryl sarcosine, and 50 µg/ml proteinase K] and incubated for 48 h at 55 °C. Plugs were then washed once with ddH_2O and twice with TE buffer [10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA] and stored at 4 °C in 10 ml TE buffer.

Restriction analysis was performed using the restriction endonucleases *Not*I and *Xho*I (Fermentas, Canada). Plugs were cut and equilibrated in 200 µl of the respective buffer for each enzyme at 4 °C for 1 h. DNA in each plug was digested with 50 U of each restriction endonuclease (2 µl enzyme, 15.5 µl buffer, 0.7 µl BSA, 0.2 µl DTT and 130 µl ddH_2O) incubated O/N at 37 °C.

DNA fragments were resolved by PFGE using a CHEF Mapper System (Bio-Rad, USA). Restriction fragments were separated in 1% agarose gels containing 0.01% thio-urea (Zhang *et al*., 2004) in 0.5× TBE buffer at 6 V cm^{-1} with a linear ramped switching time of 5-40 s for 16 h. The low range PFGE marker (New England Biolabs, USA) was used as molecular weight marker. Molecular weight of fragments was estimated using UVIDOC V.97 (UVItec, UK).

Data obtained by PFGE was evaluated according to the following criteria: typeability (percentage of distinct bacterial isolates which can be assigned a positive type), reproducibility (percentage of isolates giving the same result on repeated testing) and discrimination (ability of a typing technique to differentiate epidemiologically unrelated isolates) (Maslow and Mulligan, 1996; Tenover *et al.*, 1997). Simpson's index of diversity was used to calculate the discriminatory index of PFGE (Hunter and Gaston, 1988).

Relationships between restriction profiles were inferred using distance analysis in Phylogenetic Analysis Using Parsimony (PAUP∗) v.4.0b10 (Sinauer Associates, Sunderland, MA) and linkage distance analysis in Statistica 8 (Statsoft, Tulsa, USA). Characters were treated as unweighted in the analysis and gaps were treated as missing data. A single tree for each dataset was obtained using neighbour-joining analysis with data coded as 1 or 0 for present and absent, respectively. A bootstrap analysis (1000 replicates using the neighbour-joining option) was performed in PAUP∗ to determine the confidence levels of the nodes.

Genetically related isolates (clones) were identified according to fingerprint identity and clustering data obtained by comparison of RAPD-PCR, REP-PCR and PFGE types. Isolates sharing clonal origins were presumed to share a common parent (Tenover *et al.*, 1995).

2.2.5. Statistical Analysis

Relationships between chitinase activity, ECC, PFGE typing and degree of adherence, hydrophobicity, autoaggregation (Basson *et al.*, 2008), gliding motility, proteolytic activity as well as all other molecular typing methods (Flemming *et al.*, 2007) were determined using Pearson product moment coefficient (p values < 0.05 were considered significant) using SigmaStat V3.5 (Systat Software Inc., USA).

2.3. RESULTS

2.3.1. Chitin Degradation

The results for chitin degradation by the *F. johnsoniae*-like isolates are listed in Tables 2.2. Forty-six percent of the *F. johnsoniae*-like isolates were positive for chitin degradation within the 15 d incubation period and formed distinct, clear zones underlying or extending beyond bacterial colonies when grown on chitin-enriched EAOA (Fig. 2.1). Majority (90%) of the gliding *F. johnsoniae*-like isolates were able to degrade chitin. Most (82%) of these isolates also exhibited a hazy colonial morphology compared to the smooth colonial morphology typically associated with non-gliding strains. Only three non-gliding *F. johnsoniae*-like isolates, i.e., YO51, YO65 and YO67, displayed chitin degradation (Table 2.3).

Statistically significant positive correlations were observed between chitinase activity and motility ($r = 0.73$, $p = 0.00$) and proteolytic activity ($r = 0.61$, $p = 0.00$), respectively. Significant negative correlations were observed between chitinase activity and microtiter adherence and biofilm-forming capacity, respectively, at room temperature ($r = -0.51$, $p = 0.01$), and 26 °C ($r = -0.55$, $p = 0.00$) under static, and at room temperature ($r = -0.46$, $p = 0.01$), and 26 °C ($r = -0.61$, $p = 0.00$) under shaking conditions. Similarly, significant negative correlations were observed between chitinase activity and SAT hydrophobicity ($r = -0.62$, $p = 0.00$) and colonial morphology ($r = -0.74$, $p = 0.00$), respectively.

2.3.2. Quantification of ECC

The ECC concentration (μg/ml) of *F. johnsoniae*-like isolates ranged from 0.54 - 7.62 μg/ml for agar surface-associated and 0.2 - 1.51 μg/ml for broth cultures, respectively (Tables 2.3 and 2.4). Non-gliding, chitin-degrading isolates YO51,

YO65 and YO67 displayed ECC levels similar to that of non-gliding isolates unable to degrade chitin (Table 2.3). Only non-gliding isolate YO19, unable

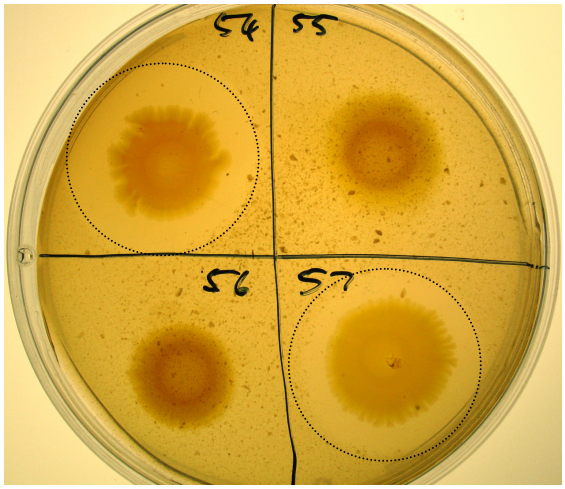

FIG. 2.1. Formation of distinct clear zones in chitin-enriched EAOA plates following a 15 d incubation period of *F. johnsoniae*-like isolates positive for chitin degradation.

to degrade chitin, demonstrated increased levels of ECC on EAOA (480 nm and 490 nm). Statistically significant positive correlations were observed between ECC (480 nm) of agar surface-associated cultures and chitinase activity (r = 0.54, p = 0.00), motility (r = 0.70, p = 0.00), proteolytic activity (r = 0.56, p = 0.00), and autoaggregation (r = 0.47, p = 0.00), respectively. Statistically significant negative correlations were observed between ECC (480 nm) of agar surface-associated cultures and microtiter adherence at room temperature (r = –0.39, p = 0.04), microtiter adherence at 26 °C (r = –0.45, p = 0.02), colonial morphology (r = –0.65, p = 0.00), SAT hydrophobicity (r = –0.55, p = 0.01), RAPD types (r = –0.47, p = 0.01), and REP types (r = –0.55, p = 0.00), respectively.

Statistically significant positive correlations were observed between ECC (490 nm) of agar surface-associated cultures and chitinase activity (r = 0.55, p = 0.00), motility (r = 0.71, p = 0.00), proteolytic activity (r = 0.57, p = 0.00), and autoaggregation (r = 0.46, p = 0.01), respectively. Statistically significant negative correlations were observed between ECC (490 nm) of agar surface-associated

TABLE 2.2. Chitin degradation of *F. johnsoniae*-like isolates cultured on chitin-enriched EAOA plates

Isolate	Average zone size (diameter in mm) over a 15 d period								
	Day 7*	8	9	10	11	12	13	14	15
YO12	1.97±0.06	2.00±0.00	2.10±0.00	2.17±0.06	2.27±0.06	2.37±0.06	2.37±0.06	2.47±0.06	2.50±0.10
YO15	1.90±0.10	1.93±0.15	2.07±0.10	2.10±0.10	2.17±0.15	2.17±0.15	2.30±0.10	2.40±0.10	2.43±0.15
YO19	-	-	-	-	-	-	-	-	-
YO20	-	-	-	-	-	-	-	-	-
YO21	-	-	-	-	-	-	-	-	-
YO26	-	-	-	-	-	-	-	-	-
YO34	-	-	-	-	-	-	-	-	-
YO35	-	-	-	-	-	-	-	-	-
YO38	-	-	-	-	-	-	-	-	-
YO45	-	-	1.83±0.10	1.87±0.06	1.97±0.06	2.07±0.06	2.07±0.06	2.07±0.06	2.07±0.06
YO49	1.70±0.10	1.83±0.06	1.83±0.06	1.93±0.06	2.00±0.00	2.07±0.06	2.10±0.00	2.20±0.00	2.30±0.00
YO50	1.70±0.00	1.80±0.00	1.83±0.06	1.90±0.00	1.97±0.06	2.03±0.06	2.13±0.06	2.20±0.00	2.27±0.06
YO51	-	-	-	-	-	1.90±0.10	1.90±0.10	1.97±0.06	1.97±0.06
YO52	-	-	-	-	-	-	-	-	-
YO53	1.60±0.10	1.67±0.06	1.83±0.06	1.93±0.06	2.00±0.00	2.00±0.00	2.07±0.06	2.13±0.06	2.17±0.06
YO54	-	1.70±0.00	1.77±0.06	1.90±0.00	1.90±0.00	2.00±0.00	2.03±0.06	2.13±0.06	2.23±0.06
YO55	-	-	-	-	-	-	-	-	-
YO56	-	-	-	-	-	-	-	-	-
YO57	-	-	-	-	-	-	-	1.67±0.15	1.90±0.10
YO59	-	-	-	-	-	-	-	-	-
YO60	-	-	1.97±0.06	2.07±0.06	2.13±0.06	2.17±0.06	2.23±0.06	2.30±0.10	2.33±0.06
YO61	-	1.50±0.10	1.60±0.10	1.67±0.06	1.77±0.06	1.83±0.06	1.93±0.06	2.00±0.00	2.07±0.06
YO62	-	-	-	-	-	-	-	-	-
YO63	-	-	-	-	-	-	-	-	-
YO64	-	-	-	-	-	-	-	-	-
YO65	-	1.10±0.10	1.20±0.10	1.33±0.06	1.53±0.06	1.73±0.06	2.07±0.10	2.17±0.15	2.30±0.20
YO66	-	-	-	-	-	-	-	-	-
YO67	-	1.17±0.10	1.20±0.10	1.43±0.06	1.63±0.06	2.03±0.06	2.43±0.10	2.60±0.10	2.67±0.06

* Zones of clearing were detected only after 7 d of incubation.

TABLE 2.3. Summary of phenotypic, physiological and molecular characterization of *F. johnsoniae*-like isolates

Isolate	Fish host	Colony Morphology*	Degree of adherence†	WCP subtypes‡	OMP subtypes§	Gliding motility¶	Casein proteolytic activity‖	Chitinase activity	Extracellular carbohydrate content		PFGE types**
								(cm ± SD)	Agar surface-ass. (480 nm)	(490 nm)	
YO12	Rainbow trout	H	W	WP1	O1	+	+	2.50 ± 0.10	2.96±0.35	2.67±0.28	NT
YO15	Rainbow trout	H	W	WP2	O2	+	+	2.43 ± 0.15	7.62±2.30	6.91±2.00	NT
YO19	Rainbow trout	S	S‡	WP3	O3	-	(+)	-	2.01±0.28	1.82±0.31	PF1
YO20	Koi	S	S	WP3	O4	-	(+)	-	1.058±0.27	0.92±0.17	PF1
YO21	Rainbow trout	S	S	WP3	O4	-	(+)	-	1.32±0.10	1.06±0.17	PF1
YO26	Rainbow trout	S	S	WP3	O4	-	(+)	-	1.56±0.23	1.33±0.21	PF1
YO34	Rainbow trout	S	S	WP3	O4	-	(+)	-	1.76±0.28	1.43±0.27	PF1
YO35	Rainbow trout	S	S	WP3	O4	-	(+)	-	1.60±0.18	1.36±0.10	PF1
YO38	Rainbow trout	S	S	WP3	O4	-	(+)	-	1.59±0.12	1.36±0.12	PF1
YO45	Rainbow trout	H	M§	WP1	O5	+	+	2.07 ± 0.06	2.50±0.45	2.20±0.19	PF2
YO49	Rainbow trout	H	W	WP1	O6	+	+	2.30 ± 0.00	2.73±0.02	2.42±0.01	PF3
YO50	Rainbow trout	H	W	WP1	O6	+	+	2.27 ± 0.06	2.09±0.03	2.04±0.05	PF3
YO51	Rainbow trout	S	W	WP4	O7	-	+	1.97 ± 0.06	1.74±0.35	1.59±0.25	PF4
YO52	Koi	S	M	WP3	O8	-	(+)	-	1.19±0.37	1.01±0.35	PF5
YO53	Koi	H	W	WP1	O9	+	+	2.17 ± 0.06	3.23±0.27	2.82±0.29	PF6
YO54	Koi	H	W	WP1	O9	+	+	2.23 ± 0.06	4.88±0.20	4.37±0.27	PF7
YO55	Koi	S	W	WP3	O10	-	-	-	0.72±0.14	0.63±0.13	PF8
YO56	Koi	S	M	WP3	O10	-	-	-	0.74±0.21	0.64±0.23	PF9
YO57	Longfin eel	S	W	WP2	O11	(+)	(+)	1.90 ± 0.10	2.13±0.20	2.02±0.18	PF10
YO59	Longfin eel	S	S	WP3	O12	-	+	-	0.57±0.28	0.54±0.05	PF11
YO60	Longfin eel	H	W	WP2	O11	(+)	+	2.33 ± 0.06	2.18±0.39	1.93±0.28	PF12
YO61	Longfin eel	H	W	WP2	O13	+	+	2.07 ± 0.06	2.35±0.35	2.15±0.32	PF13
YO62	Longfin eel	S	W	WP2	O11	(+)	+	-	3.08±0.35	2.89±034	PF14
YO63	Longfin eel	S	S	WP3	O14	-	(+)	-	0.61±0.16	0.54±0.08	PF15
YO64	Longfin eel	S	S	WP3	O14	-	(+)	-	0.90±0.21	0.75±0.26	PF15
YO65	Biofilm	S	W	WP4	O15	-	(+)	2.30 ± 0.20	1.28±0.37	1.13±0.31	PF16
YO66	Biofilm	S	M	WP3	O16	-	(+)	-	1.41±0.18	1.24±0.16	PF17
YO67	Biofilm	S	W	WP4	O15	-	+	2.67 ± 0.06	1.36±0.26	1.17±0.25	PF18

* Hazy colonial morphology (H), smooth colonial morphology (S) (Flemming *et al*., 2007).

† Weakly adherent (W); Strongly adherent (S); Moderately adherent (M) - adherence was determined following growth in EAOB at 26 °C (Basson *et al*., 2008).

‡ WP1-WP4 represent whole cell protein profile subtypes (Flemming *et al*., 2007).

§ O1-O16 represent outer membrane protein profile subtypes (Flemming *et al*., 2007).

¶ + Strong gliding ability; (+) weak gliding ability; - non-gliding isolates (Flemming *et al*., 2007).

‖ + Strong casein proteolytic activity; (+) weak casein proteolytic activity (Flemming *et al*., 2007).

** NT refers to not typeable since *Xho*I PFGE typing resulted in a DNA smear.

cultures and microtiter adherence at room temperature (r = −0.39, p = 0.04), microtiter adherence at 26 °C (r = −0.45, p = 0.02), SAT hydrophobicity (r = −0.56, p = 0.01), RAPD types (r = −0.45, p = 0.02), and REP types (r = −0.53, p = 0.00), respectively.

The results obtained for ECC from broth cultures were more uniform and lower compared to the levels of ECC formed on EAOA for all *F. johnsoniae*-like isolates tested (Table 2.4). Only isolate YO15 displayed increased levels of ECC in EAOB, which in general were much lower compared to the level of ECC formed on EAOA (Table 2.4). Statistically significant positive correlations were observed between ECC (480 nm) of broth cultures and motility (r = 0.39, p = 0.04), proteolytic activity (r = 0.51, p = 0.01), and autoaggregation (r = 0.57, p = 0.00), respectively. Statistically significant negative correlations were observed between ECC (480 nm) of broth cultures and RAPD types (r = −0.45, p = 0.02), and REP types (r = −0.46, p = 0.01), respectively.

Statistically significant positive correlations were observed between ECC (490 nm) of broth cultures and proteolytic activity (r = 0.49, p = 0.01), and autoaggregation (r = 0.60, p = 0.00), respectively. Statistically significant negative correlations were observed between ECC (480 nm) of broth cultures and RAPD types (r = −0.38, p = 0.05), and REP types (r = −0.39, p = 0.04), respectively.

2.3.3. Restriction Analysis with PFGE

Restriction of genomic DNA with *Not*I yielded insufficient band fragments and fragment sizes upon electrophoresis. Using *Xho*I fingerprints and visual analysis, study isolates could be differentiated into 18 types (PF1-18) (Tables 2.1 and 2.3; Fig. 2.2 A-C). Fingerprints consisted of 10 - 17 of fragments in the 6.55 - 273 kb size range (Table 2.5). Approximately 93% of the *F. johnsoniae*-like isolates produced a visible PFGE pattern with *Xho*I. Two isolates, YO12 and YO15, were

TABLE 2.4. **Extracellular carbohydrate content of *F. johnsoniae*-like isolates grown in the agar surface-associated and planktonic phases**

Isolate	Agar surface-associated cultures		Broth cultures	
	480 nm	490 nm	480 nm	490 nm
YO12	2.96±0.35	2.67±0.28	0.58±0.00	0.40±0.00
YO15	7.62±2.30	6.91±2.00	1.51±0.08	1.16±0.07
YO19	2.01±0.28	1.82±0.31	0.48±0.01	0.40±0.00
YO20	1.06±0.27	0.92±0.17	0.41±0.00	0.41±0.00
YO21	1.32±0.10	1.06±0.17	0.58±0.02	0.45±0.01
YO26	1.56±0.23	1.33±0.21	0.57±0.03	0.47±0.03
YO34	1.76±0.28	1.43±0.27	0.57±0.01	0.46±0.00
YO35	1.60±0.18	1.36±0.10	0.58±0.02	0.47±0.01
YO38	1.59±0.12	1.36±0.12	0.58±0.04	0.48±0.00
YO45	2.50±0.45	2.20±0.19	0.69±0.05	0.58±0.06
YO49	2.73±0.02	2.42±0.01	0.38±0.00	0.26±0.00
YO50	2.09±0.03	2.04±0.05	0.56±0.00	0.37±0.00
YO51	1.74±0.35	1.59±0.25	0.46±0.01	0.36±0.00
YO52	1.19±0.37	1.01±0.35	0.48±0.00	0.36±0.00
YO53	3.23±0.27	2.82±0.29	0.46±0.03	0.29±0.01
YO54	4.88±0.20	4.37±0.27	0.45±0.00	0.43±0.01
YO55	0.72±0.14	0.63±0.13	0.20±0.00	0.21±0.00
YO56	0.74±0.21	0.64±0.23	0.20±0.00	0.18±0.00
YO57	2.13±0.20	2.02±0.18	0.76±0.03	0.65±0.01
YO59	0.57±0.28	0.54±0.05	0.31±0.00	0.28±0.00
YO60	2.18±0.39	1.93±0.28	0.47±0.01	0.39±0.00
YO61	2.35±0.35	2.15±0.32	0.32±0.00	0.29±0.00
YO62	3.08±0.35	2.89±034	0.55±0.01	0.50±0.01
YO63	0.61±0.16	0.54±0.08	0.25±0.00	0.23±0.00
YO64	0.90±0.21	0.75±0.26	0.37±0.00	0.33±0.00
YO65	1.28±0.37	1.13±0.31	0.36±0.00	0.33±0.00
YO66	1.41±0.18	1.24±0.16	0.38±0.00	0.32±0.00
YO67	1.36±0.26	1.17±0.25	0.45±0.01	0.41±0.01

* ECC measured in μg/ml.

untypeable, since *Xho*I digestion resulted in a DNA smear in the region of ~ 48 kb (Fig 2.2 A, Lanes 1 - 2). Despite the 6% formaldehyde treatment, restriction endonuclease digestion of these two isolates remained a smear following visualization. Since not all the isolates were typeable with restriction analysis and PFGE, the typeability index of PFGE was 92.8%. Using the Simpson's index of diversity, the discriminatory index of PFGE was 0.929 (92.9%).

Identical PFGE fingerprints were observed for isolates YO19-YO38, YO49-YO50 and YO63-YO64, respectively (Table 2.5; Fig. 2.2 A-C). Fragments

of approximately 77 and 57.3 kb, respectively, were present in all profiles PF1-18 (Table 2.5). Fragments of approximately 105 kb and 91 kb were present in 78.6% and 89% of fingerprints, respectively. A fragment of approximately 12.1 kb was present only in fingerprints of PFGE types PF5-9, representing isolates obtained from a disease outbreak in Koi (Stellenbosch, 2004) (Table 2.3).

Cluster analysis of *Xho*I fingerprints using PAUP* (Fig. 2.3) divided the isolates into two major groups, A and B, with group A being further subdivided into 4 subgroups and group B consisting of 3 subgroups. Bootstrap values of groups ≥85% were considered to represent isolates with high genetic relatedness. Linkage distance analysis of *Xho*I fingerprints using Statistica 8 (Statsoft, Tulsa, USA) divided the isolates into two major groups, A and B, at >50% similarity, with group A further subdivided into 7 subgroups and group B consisting of 5 subgroups (Fig. 2.4). PFGE types were grouped similarly within the two major groups with cluster analysis using PAUP* and linkage distance using Statistica 8, respectively, with the PFGE type PF3 (isolates YO49 and YO50) being the only exception (Figs. 2.3 and 2.4). Subgroups of isolates considered to have possible clonal ancestors included isolates belonging to PFGE types PF1 (100% similarity), PF3 (100% similarity), PF5 + PF8 + PF9 (>90% similarity), PF6 + PF7 (>90% similarity), PF15 (>90% similarity), respectively (Fig. 2.4).

From Tables 2.1 and 2.3, it could be distinctly observed that isolates with PFGE profiles PF1, PF5 + PF9, PF11 + PF15, displayed high genetic similarity, a strong biofilm phenotype and the W3 whole cell protein profile, respectively. The *F. johnsoniae*-like isolates obtained from diseased Koi (PF5-9) formed a subgroup in group A with a bootstrap value of 60% using PAUP* (Fig. 2.3). Overall, these isolates were also grouped at >78% similarity, and further divided into two groups at >90% similarity with linkage distance using Statistica 8 (Fig. 2.4). Many of the profiles deemed identical by visual analysis did not cluster closely together using PAUP*. This would be a result of default algorithm parameters preset in the PAUP* software package.

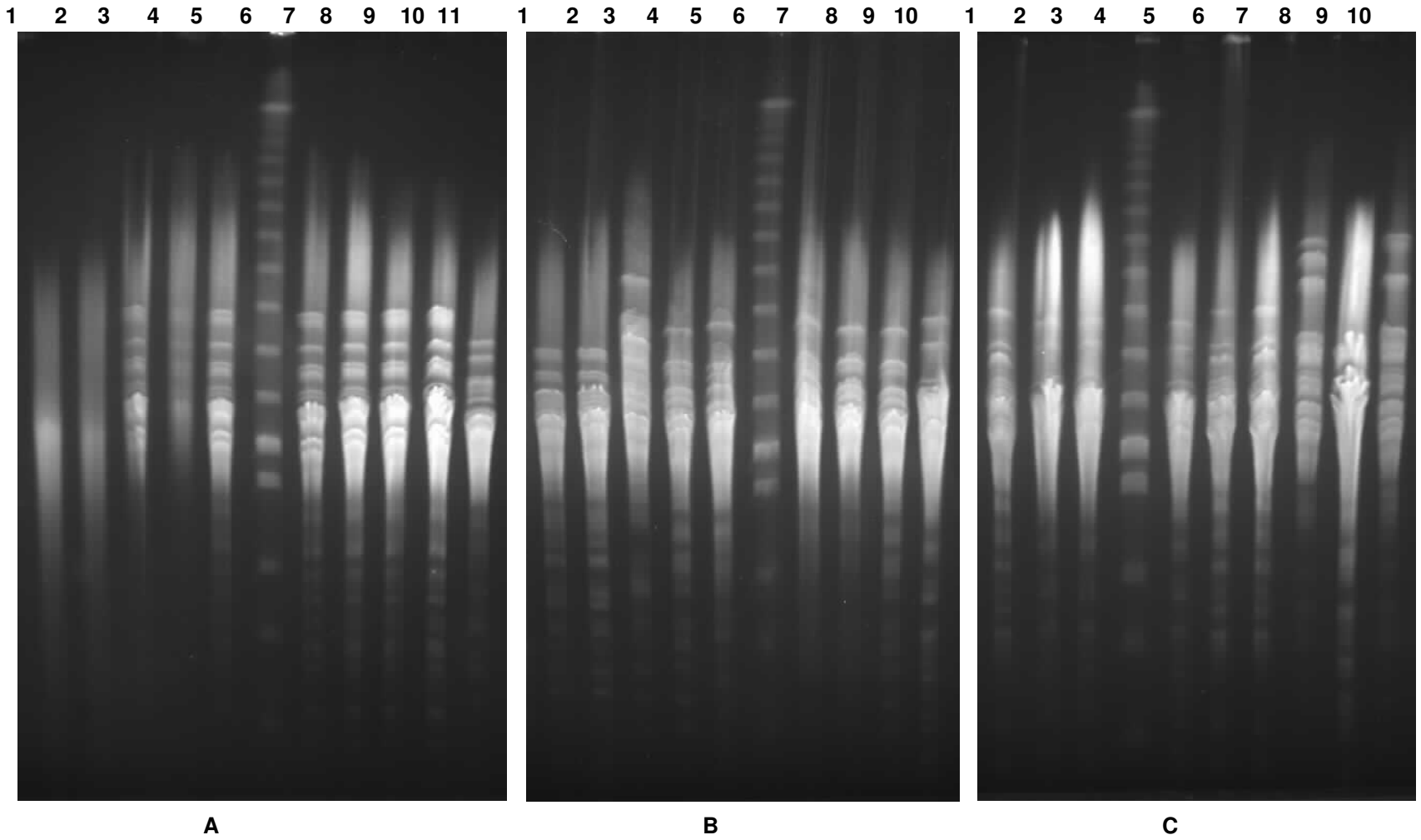

Fig. 2.2. PFGE profiles obtained with *Xho*I-digested genomic DNA from *F. johnsoniae*-like isolates. Lanes A6, B6 and C4: Low range PFGE marker (194.0/ 145.5/ 97.0/ 48.5/ 23.1/9.42/6.55/4.36/2.32/ 2.03 kb; New England Biolabs, USA); lanes A1-5 and A7-11: isolates YO12, YO15, YO19, YO20, YO21 and YO26, YO34, YO35, YO38, and YO45, respectively; lanes B1-5 and B7-10: isolates YO49, YO50, YO51, YO52, YO53 and YO54, YO55, YO56 and YO57, respectively; and lanes C1-3 and C5-10: isolates YO59, YO60, YO61 and YO62, YO63, YO64, YO65, YO66, and YO67, respectively.

Table 2.5. Number and molecular weight of restriction fragments obtained following *Xho*I and PFGE analysis of *F. johnsoniae* genomic DNA

Isolate	YO12	YO15	YO19	YO20	YO21	YO26	YO34	YO35	YO38	YO45	YO49	YO50	YO51	YO52	YO53	YO54	YO55	YO56	YO57	YO59	YO60	YO61	YO62	YO63	YO64	YO65	YO66	YO67
Subtype*			PF1							PF2	PF3		PF4	PF5	PF6	PF7	PF8	PF9	PF10	PF11	PF12	PF13	PF14	PF15		PF16	PF17	PF18
Size (kb)																												
																												273
																										269		
																												262
																										249		
																												227
													221													221		
			192	192	192	192	192	192	192											192				192	192			
			184	184	184	184	184	184	184								184		184		184	184	184					
															183	183												
														171			171	171										171
																										164		
			155	155	155	155	155	155	155	155			155						155	155	155	155	155	155	155	155	155	155
											149	149																
			141	141	141	141	141	141	141	141			141	141	141	141	141	141		141				141	141	141	141	141
			135	135	135	135	135	135	135				135	135	135	135	135	135										135
										128	128	128			128	128				128				128	128	128	128	128
			127	127	127	127	127	127	127	127					127	127						127	127	127	127			
			105	105	105	105	105	105	105		105	105	105	105			105	105	105	105	105	105		105	105	105	105	105
										97																		
			91	91	91	91	91	91	91		91	91	91	91	91	91	91	91	91	91	91	91	91	91	91	91	91	91
										79.8																		
			77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77	77
																										60		60
			57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3	57.3
	48	48											48															
										35	35	35														35		35
			30.5	30.5	30.5	30.5	30.5	30.5	30.5					30.5	30.5		30.5	30.5	30.5					30.5	30.5			
										29																		
														17.1	17.1			17.1	17.1	17.1	17.1	17.1	17.1	17.1	17.1			
			15	15	15	15	15	15	15		15	15	15						15								15	
														12.1	12.1	12.1	12.1	12.1										
											11.5	11.5																
			10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8					10.8				10.8		10.8	10.8	10.8					
																											10.1	
											10	10		10														
			9.42	9.42	9.42	9.42	9.42	9.42	9.42					9.42	9.42	9.42	9.42	9.42		9.42				9.42	9.42	9.42	9.42	9.42
													9															
											8.9	8.9		8.9	8.9			8.9	8.9	8.9	8.9	8.9	8.9	8.9	8.9			
			8.2	8.2	8.2	8.2	8.2	8.2	8.2		8.2	8.2	8.2							8.2						8.2		8.2
																				7.8				7.8	7.8		7.8	
													7.6															
			7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2									7.2	7.2	7.2					
															6.8	6.8			6.8	6.8				6.8	6.8			
																											6.6	
			6.55	6.55	6.55	6.55	6.55	6.55	6.55																			
No. of fragments	1	1	17	17	17	17	17	17	17	12	13	13	13	13	15	11	11	12	12	14	10	11	10	15	15	15	12	16

* PF1 - PF18 refers to subtypes identified following *Xho*I-PFGE typing.

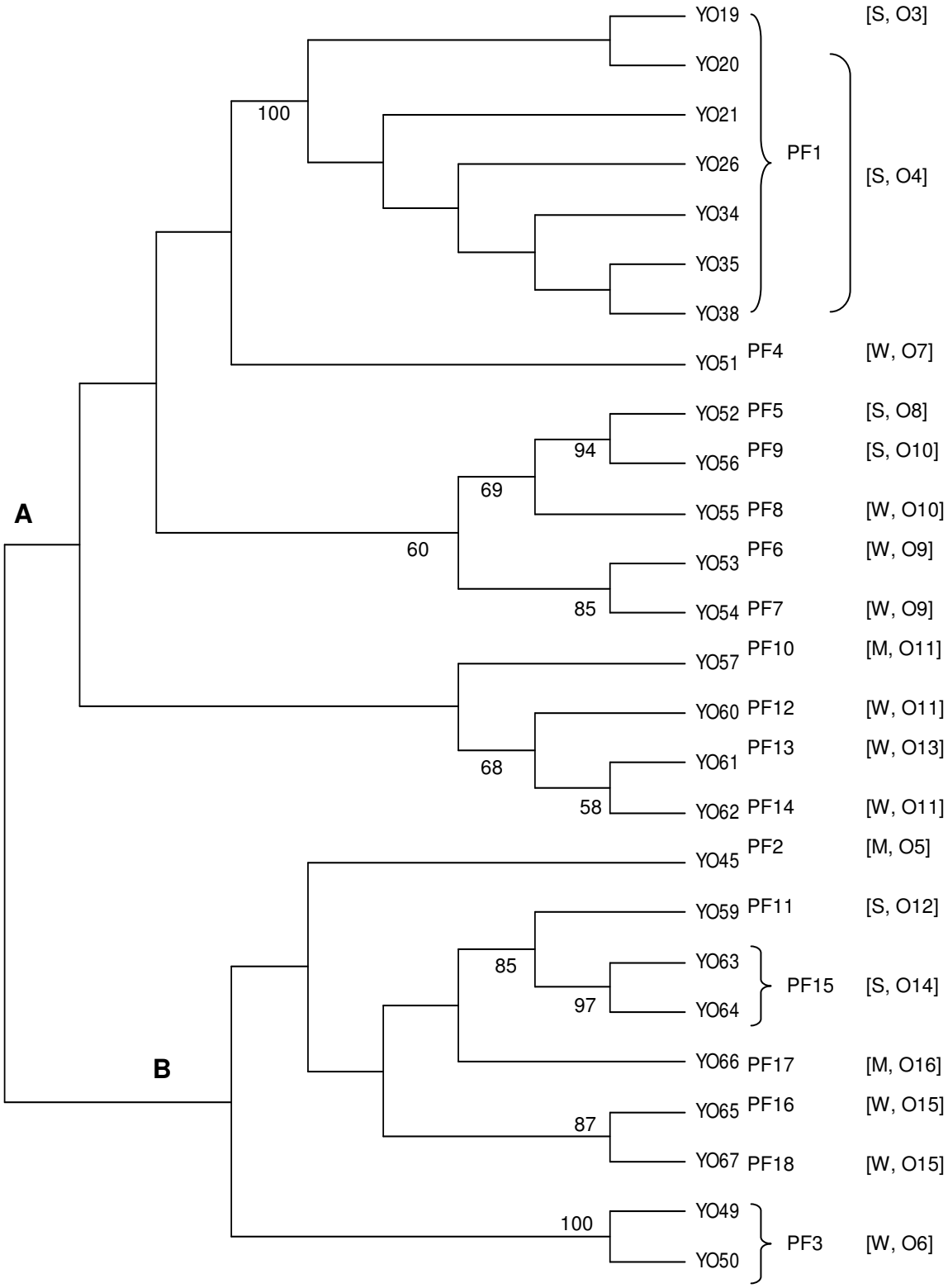

Fig. 2.3. Dendrogram of PFGE profiles (PF1-PF18) of *Flavobacterium johnsoniae*-like isolates generated with PAUP∗ by cluster analysis using Parsimony and Bootstrap cluster analysis. S (strongly adherent), W (weakly adherent), and M (moderately adherent) refer to biofilm phenotypes and O3 - O16 refer to OMP subtypes (Flemming *et al*., 2007).

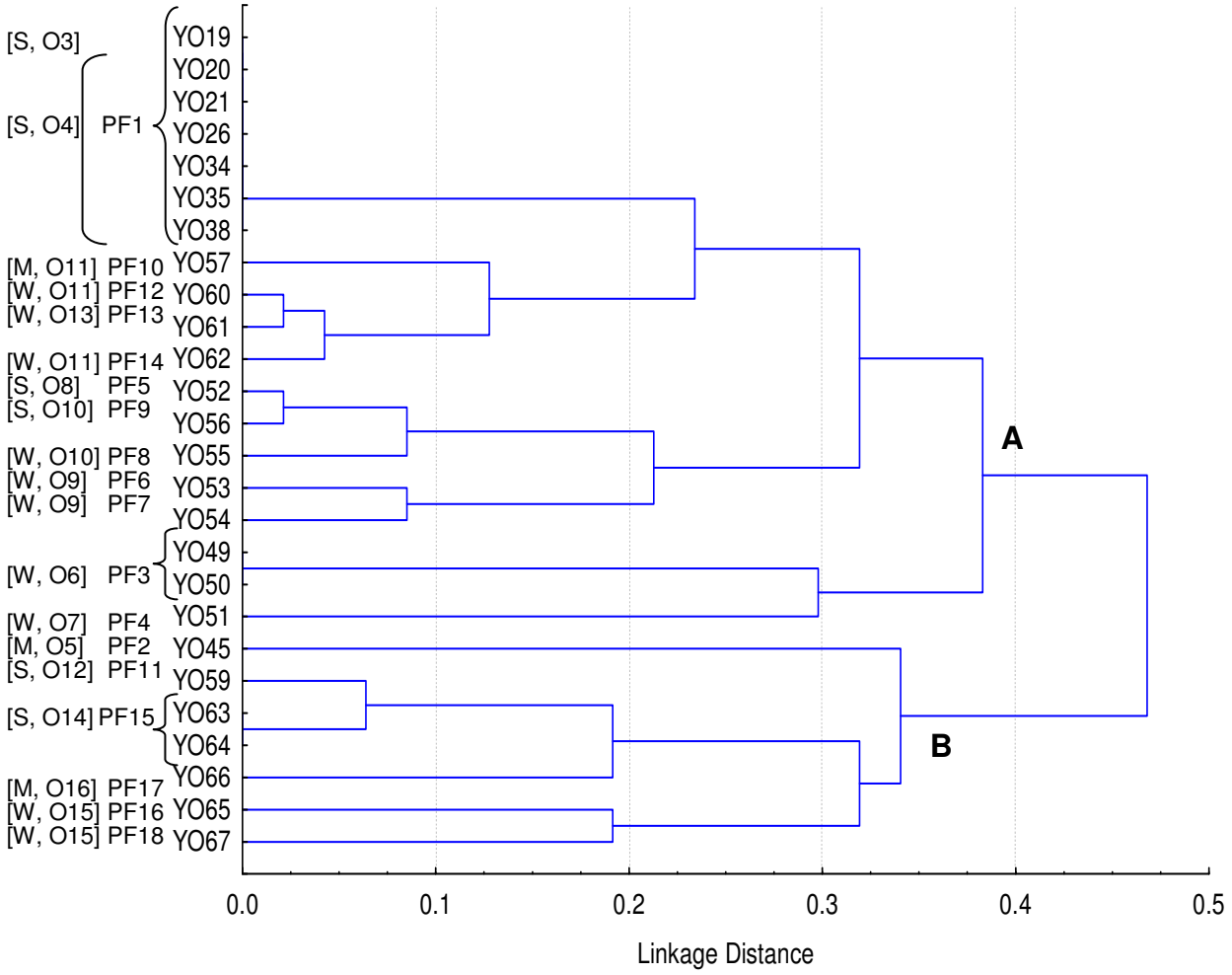

Fig. 2.4. Dendrogram of PFGE profiles (PF1-PF18) of *Flavobacterium johnsoniae*-like isolates generated with Statistica by Cluster Analysis. Isolates belonging to PFGE profiles with 0% linkage distance (100% similarity) were considered to have possible clonal origins. S (strongly adherent), W (weakly adherent), and M (moderately adherent) refer to biofilm phenotypes and O3 - O16 refer to OMP subtypes (Flemming *et al.*, 2007).

F. johnsoniae-like isolates displaying the specific PFGE types PF1, PF3, and PF15 also shared the same RAPD, REP and OMP subtypes (Table 2.1). All isolates displaying the specific PFGE type PF1 also displayed the smooth colonial morphology (Table 2.3). Statistically significant positive correlations were observed between PFGE and RAPD-PCR analysis (r = 0.90, p = 0.00), REP-PCR analysis (r = 0.84, p = 0.00), WCP analysis (r = 0.49, p = 0.01), OMP analysis (r = 0.90, p = 0.00), and fish host (r = 0.95, p = 0.00) respectively. Majority of non-gliding *F. johnsoniae*-like isolates which lacked chitinase activity, also produced low amounts of ECC on EAOA. Additionally, they displayed the W3 WCP subtype, a strong biofilm phenotype and showed no or decreased (weak) casein-proteolytic activity (Table 2.3). Only non-gliding isolates YO51, YO65 and YO67, belonging to the W4 WCP subtype, displayed a weak biofilm phenotype and chitinase activity, respectively. All motile *F. johnsoniae*-like isolates displayed chitinase activity, produced high amounts of ECC on EAOA, displayed variable WCP profiles, had weak to moderate biofilm phenotypes and showed strong casein-proteolytic activity (Table 2.3). None of the RAPD and/or REP types could be linked to chitin degradation and significant negative correlations were observed between RAPD and/or REP types and ECC production on EAOA and EAOB. A statistically significant negative correlation was observed between PFGE and ECC (480) of broth cultures (r = -0.45, p = 0.02).

2.1. DISCUSSION

Chitin plays an important role in the transition between the multi-cellular sessile and free-living organotrophic lifestyle and influences the pathogenicity of certain aquatic pathogens (Pruzzo *et al*., 2008). In *V. cholerae*, chitin induces biofilm formation and DNA transformation providing a mechanism whereby this aquatic pathogen adapts to the aquatic environment and infects the human host (Nalin *et al*., 1979; Pruzzo *et al*., 2008; Reguera and Kolter, 2005). There is evidence that

chitin-associated virulence factors required for infection play an important role in biofilm formation, and *vice versa* (Pruzzo *et al*., 2008; Reguera and Kolter, 2005). In *V. cholerae*, the presence of chitin and chitin oligomers stimulates the production of type IV pili required for adherence and DNA transfer and components of the general secretory pathway necessary for the transport of chitinases and cholera toxin (Pruzzo *et al*., 2008). The *V. cholerae* toxin-corregulated pilus, necessary for intestinal colonization and cholera toxin acquisition, mediates cellular interactions required for biofilm differentiation on chitinaceous surfaces (Reguera and Kolter, 2005). Virulent *V. cholerae* strains colonize chitinaceous surfaces and chitinous organisms as part of an adaptive mechanism to survive in the aquatic environment leading to cholera outbreaks when introduced to hosts (Lipp *et al*., 2002; Reguera and Kolter, 2005).

Since *F. johnsoniae* is a soil-based organotroph capable of degrading complex macromolecules as part of its requirement to gain energy (Bernardet and Bowman, 2006), one would expect to frequently isolate environmental isolates with the ability to degrade biopolymers, such as chitin. However, according to the results obtained in this study, only 43% of wild-type *F. johnsoniae*-like isolates obtained from diseased fish were able to degrade chitin.

In *F. johnsoniae*, chitin degradation is associated with gliding motility and a loss in motility leads to a loss in chitin degradation (McBride, 2004). The chitin-degrading and gliding ability of *F. johnsoniae* may play a role in the virulence of this organism. *F. johnsoniae*-like isolates classified as most virulent following an infection study were motile, displayed the hazy colonial morphology, displayed increased casein proteolytic activity and chitin degradation, and weak adherence to polystyrene compared to non- or low-virulent isolates (Flemming *et al*., 2007). By contrast, non- or low-virulent *F. johnsoniae*-like isolates displayed weak casein proteolytic activity, no chitin degradation, were non-motile, displayed the smooth colonial morphology and strong adherence to polystyrene. An increased biofilm-forming capacity of pathogenic organisms, such as *V. cholerae*, is frequently associated with their pathogenicity (Reguera and Kolter, 2005). However, for members of the genus *Flavobacterium*, virulence and/or chitin

degradation and biofilm formation are antagonistic properties. A *F. psychrophilum* non-motile mutant was deficient not only in extracellular proteolytic activity and cytotoxicity, but also lost virulence (Álvarez *et al*., 2006). The *F. psychrophilum* non-motile mutant deficient in virulence displayed enhanced biofilm growth (Álvarez *et al*., 2006). A similar gliding motility/virulence relationship was observed for *F. columnare* (Kunttu *et al*., 2009). In *F. columnare*, a strong adhesion capacity was not correlated with gliding motility or virulence. Instead, colonial morphology was suggested to influence adherence and virulence in *F. columnare* (Kunttu *et al*., 2009). A non-rhizoid and hard colony type was associated with increased adherence to polystyrene, while a specific rhizoid colony type was suggested to determine virulence in *F. columnare* (Kunttu *et al*., 2009). For *V. cholerae*, a rugose colony type, which is also associated with virulence, is correlated with biofilm formation (Beyhan *et al*., 2007). In the present study, significant negative correlations were observed between motility/chitinase activity and biofilm formation on polystyrene at both room temperature and 26 °C, respectively. Similar to *F. columnare* (Kunttu *et al*., 2009) and *V. cholerae* (Beyhan *et al*., 2007), colony phase variation between the hazy and smooth colony types of *F. johnsoniae*-like isolates may determine and/or influence their virulence and biofilm-forming ability. Hence, different adhesion factors of *F. johnsoniae*-like isolates may be involved in the attachment processes to polystyrene (abiotic) and fish tissue or chitinaceous surfaces (biotic), respectively, which may also determine virulence.

McBride (2004) described the existence of motile non-gliding *F. johnsoniae* mutants, which were able to glide in wet mounts, but unable to spread on agar surfaces. This led to the identification of SprA, a large OMP partially exposed on the cell surface, required for cell adhesion to glass, polystyrene and Teflon surfaces, colony spreading and efficient cell movement (Nelson *et al*., 2007). However, the exact function of SprA in gliding and bacterial adherence remains unclear. Non-spreading *F. johnsoniae*-like isolates YO51, YO65 and YO67 with the ability to degrade chitin may still be motile in wet mounts. The *sprA* mutants described by Nelson *et al*. (2007) were also only partially defective

in chitin utilization. The non-spreading *F. johnsoniae*-like isolates YO51, YO65 and YO67 have previously demonstrated variable WCP and OMP profiles compared to other non-gliding isolates unable to degrade chitin (Table 2.3). This might be related to the production of proteins and enzymes, such as SprA, chitinase and chitin-binding proteins, involved in gliding, adhesion, chitin degradation and transport of chitin across the cell membrane. Furthermore, mutations in the *gldA*, *gldF* and *gldG* genes coding for a probable transmembrane ABC transporter have been shown to affect both the gliding and chitin utilization abilities of *F. johnsoniae* (McBride and Braun, 2004; McBride *et al*., 2003). ABC transporters are known to export polysaccharides and EPS which form part of capsular material and also play a role in bacterial adherence and subsequent biofilm formation (Haft *et al*., 2006; Norton *et al*., 2008; Silver *et al*., 2001). Additionally, SusC and SusD-like proteins, which are outer membrane polysaccharide utilization proteins, have been suggested to play a role in chitin utilization of *F. johnsoniae* (McBride *et al*., 2009). These proteins may also be responsible for the chitin-degrading abilities of *F. johnsoniae*-like isolates. Differences observed in colonial morphology for gliding (hazy) and non-gliding (smooth) isolates may be explained by these differences in protein production (Flemming *et al*., 2007), as well as possible differences in the production or excretion of cell surface polysaccharides as observed in *V. cholerae* rugose and smooth colonial variants (Beyhan *et al*., 2007).

Cell surface polysaccharide, including carbohydrate components in the capsule layer, and the production of EPS are also associated with biofilm formation (Beyhan *et al*., 2007; Decostere *et al*., 1999a; Donlan, 2002; Hall-Stoodley and Stoodley, 2002). Excessive EPS production by *Flavobacterium* spp. has been associated with paper spoilage and this characteristic of biofilm-forming organisms remains a problem in blockage of pipes in the commercial industry (Oppong *et al*., 2003). EPS may play an important role in establishing biofilm growth by enhancing attachment and microcolony formation and/or by forming part of the matrix that supports the mature biofilm structure (Sutherland, 2001), which may explain the higher concentrations of ECC present in agar

surface-associated cultures compared to broth cultures of *F. johnsoniae*-like isolates (Table 2.4). However, variation in ECC results from broth and plate assays may also be explained by a loss in extracellular material from ECC fraction I in the broth medium, rather than a lack of ECC production (Ryu and Beuchat, 2003). Most of the exopolysaccharides present in planktonic cultures are lost during centrifugation, only capsular polysaccharide remains cell-associated (Branda *et al*., 2005). The ECC measured for planktonic *F. johnsoniae*-like isolates may represent capsular polysaccharides. Using a similar EPS isolation technique, Kives *et al*. (2006) also obtained low recovery rates of planktonic EPS material, which did not allow for accurate comparison with biofilm EPS. Although the technique used in this study is not recommended for comparison of broth and biofilm ECC, it may still be useful for the quantification of capsular polysaccharides in planktonic cells.

Polysaccharide function may be related to the composition and quantity of the capsular material or EPS expressed (Joseph and Wright, 2004; Kives *et al*., 2006). Compositional differences have been reported in planktonic and biofilm EPS (Kives *et al*., 2006). Variation in EPS composition was also demonstrated to be affected by different substrate surfaces (Kives *et al*., 2006; Ledeboer and Jones, 2005). In *Salmonella enterica*, biofilm formation on intestinal tissue was disrupted in mutant cells lacking colonic acid production, but biofilm formation on a plastic surface remained unaffected (Ledeboer and Jones, 2005). Mutations in cellulose biosynthesis in turn, disrupted biofilm formation on both biotic and abiotic surfaces (Ledeboer and Jones, 2005). Differences observed in the absorbance of the *F. johnsoniae*-like isolates S-1 and S-2 taken at 480 nm and 490 nm, respectively, may be due to the presence of different polysaccharides and sugars in these fractions. Pentoses, methylpentoses and uronic acid have an absorption maximum at 480 nm, whereas hexoses and methyl derivatives of hexoses have an absorption maximum at 485-490 nm (Dubois *et al*., 1956). Furthermore, the differences observed in ECC for planktonic and agar surface-associated *F. johnsoniae*-like cells may be due to compositional differences, which may be related to differential expression and/or function in the two different

growth phases (Branda *et al*., 2005; Kives *et al*., 2006; Sauer *et al*., 2002; Sutherland, 2001). ECC of agar surface-associated cells may play an important role in the physicochemical properties of *F. johnsoniae*-like biofilms. Acetylation of uronic acids, such as polyguluronic acid, or alginate lacking guluronic acid residues may increase the viscoelastic properties of biofilms, while hexoses and hexose residues, which are more rigid and robust, increase the gel-like matrix (Sutherland, 2001). Hexose polymers, such as glucan, may act as intramolecular bridges by binding lectins on the substrate surface (Sutherland, 2001), while rhamnolipids may be involved in the biofilm channel structure (Kives *et al*., 2006). Uronic acids have also been found to be the main carbohydrate components of planktonic ECC and may represent capsular polysaccharide (Kives *et al*., 2006). The higher concentration of ECC measured at 480 nm may be directly related to the presence of uronic acids in capsules of *F. johnsoniae*-like isolates. In *V. vulnificus*, uronic acid sugars present in capsule inhibited biofilm formation and aggregation, but contributed to negative charge and ironically, hydrophobicity (Joseph and Wright, 2004), a physicochemical property frequently associated with enhanced autoaggregation, bacterial attachment and subsequent biofilm formation (Donlan, 2002; Rickard *et al*., 2004).

Thick capsule may also mask cell surface adhesins, such as pili (Donlan, 2002) or the cell surface filaments present in *F. johnsoniae* (Liu *et al*., 2007), necessary for attachment. Interestingly, a statistical significant negative correlation was observed between the production of ECC in the agar surface-associated phase of *F. johnsoniae*-like isolates and biofilm formation on polystyrene, while significant positive correlations were observed between the production of ECC in the agar surface-associated phase and casein proteolytic activity and chitin degradation, respectively. It appears that high virulent *F. johnsoniae*-like isolates have thicker capsules, which may prevent the attachment of these isolates to abiotic surfaces, such as polystyrene. High virulent strains of *F. columnare* (Decostere *et al*., 1999a) and *F. psychrophilum* (Møller *et al*., 2003), respectively, display thick capsule containing glycoproteins necessary for adherence to fish tissue (Decostere *et al*., 1999a; Møller *et al*., 2003). More

virulent *F. johnsoniae*-like isolates with increased ECC, representing thicker capsule, may attach more readily to fish tissue and other biotic surfaces, while less virulent strains with thinner capsule may use cell surface filaments and/or other protein structures to attach to abiotic surfaces, displaying a strong biofilm phenotype on Perspex and polystyrene surfaces (Basson *et al*., 2008).

The production of cell surface polysaccharides and carbohydrate has also been shown to affect colony spreading of *F. johnsoniae* (Godchaux *et al*., 1991). Mutant strains with less cell surface polysaccharide formed colonies that spread less than wild-type strains, however, they still retained some ability to glide (Godchaux *et al*., 1991). Increased amounts of ECC may also be related to gliding motility of the *F. johnsoniae*-like isolates. Gliding isolates produced higher amounts of ECC on EAOA (Table 2.3), which correlates with observations by Godchaux *et al*. (1991) that wild-type *F. johnsoniae* strains with a spreading ability on agar produced more cell surface polysaccharides. Statistically significant positive correlations were also observed between ECC (480 nm and 490 nm, respectively) of agar surface-associated cultures and gliding motility. Exopolysaccharides produced by *F. johnsoniae* may coat the substratum to optimize attachment through cell-surface adhesins, such as SprB and SprB-like proteins, to facilitate cell movement over diverse surfaces (McBride *et al*., 2009). The increased ECC observed for gliding *F. johnsoniae*-like isolates may also form part of conditioning films facilitating adhesion and cell movement on the agar surface.

Autoaggregation, i.e., process by which genetically identical bacteria attach to one another via highly specific protein and/or polysaccharide adhesins (Rickard *et al*., 2003), is an important factor in the establishment of surface aggregates and microcolonies of many bacteria (Hall-Stoodley and Stoodley, 2002; Van Houdt and Michiels, 2005; Wang *et al*., 2004b). Neutral sugars, such as glucose and galactose, predominate in *V. cholerae* EPS, aiding in autoaggregation, adherence and biofilm formation (Yildiz and Schoolnik, 1999). A statistically significant positive correlation was observed between the production of ECC in the agar surface-associated phase and autoaggregation of

F. johnsoniae-like isolates in this study. The *F. johnsoniae*-like isolates have also been classified as moderately hydrophobic to very hydrophilic (Basson *et al*., 2008), which may indicate the presence of neutral sugars in ECC. Hence, EPS production in these *F. johnsoniae*-like isolates may enhance autoaggregation required for irreversible attachment and establishing microcolonies. Although the exact sugar content of ECC fractions I and II of *F. johnsoniae*-like isolates is not known, the different carbohydrate components, i.e., charged sugars and neutral sugars, may contribute to autoaggregation, microcolony formation and biofilm structure and developmental processes in *F. johnsoniae*-like biofilm formation (Yildiz and Schoolnik, 1999).

In epidemiological typing studies, the characterization and determination of relatedness among bacterial isolates obtained from infectious disease outbreaks is essential (Gautom, 1997; Tenover *et al*., 1995). PFGE has been used successfully as an epidemiologic tool to identify clonal origins of clinically important and environmental strains of *Aeromonas* spp. (García *et al*., 2000; O'hIci *et al*., 2000; Talon *et al*., 1996). With the use of RAPD-PCR and PFGE, O'hIci *et al*. (2000) observed that the fish pathogenic species *A. salmonicida* is comprised of a genetically heterogeneous group confined to a specific clonal lineage. In the present study, *F. johnsoniae*-like isolates obtained from diseased fish in South Africa displayed genetic heterogeneity when differentiated by PFGE and six groups with possible clonal origins were identified (Figs. 2.3 and 2.4). The isolates displaying high genetic similarity, with possible clonal origins, may have common origins. Although a significant positive correlation was observed between PFGE types and fish-host species, *F. johnsoniae*-like isolates (YO20 and YO52-YO56) obtained from diseased Koi formed distinct subgroups with cluster analysis and linkage distance analysis, displaying 6 different PFGE types, viz., PF1 and PF5-PF9 (Figs. 2.3 - 2.4).

Arai *et al*. (2007) observed considerable genetic heterogeneity (42 genotypes) amongst 64 *F. psychrophilum* isolates typed by PFGE, but no clonal lineage could be established. The majority of these isolates, obtained from ayu, fell within distinct clusters, which clearly differed from genotypes observed for *F.*

psychrophilum isolates obtained from other fish species (Arai *et al*., 2007). Their results using PFGE for differentiating amongst *F. psychrophilum* isolates were found to be superior to 16S rRNA gene PCR-RFLP analysis. Similarly, PFGE typing of *F. johnsoniae*-like isolates using *Xho*I in the present study was superior to PCR-RFLP discrimination of 16S rRNA genes (Flemming *et al*., 2007).

In comparison to previously used typing methods (Flemming *et al*., 2007), isolates grouped by PFGE corresponded to clusters obtained by RAPD fingerprinting (Table 2.1). With the exception of isolates YO19-YO38, all other isolates with identical REP and RAPD fingerprints (except YO12-YO15) were further differentiated using *Xho*I and PFGE (Table 2.1). Hence, based on the improved discrimination of isolates clustered together by RAPD-PCR, REP-PCR and PFGE, it can be recommended that a combination of PCR-based typing and PFGE be used for the differentiation and discrimination of *F. johnsoniae*-like isolates. Sufficient genetic heterogeneity exists amongst the *F. johnsoniae*-like isolates used in the present study to support the use of PFGE as epidemiologic tool for the analysis of disease outbreaks caused by these organisms and this technique may also be valuable for clonal identification of *F. johnsoniae*-like isolates.

Soto *et al*. (2008) reported *F. columnare* to produce indistinguishable DNA profiles and/or smears, using standard PFGE methods, due to DNase activity. Despite an increased amount (6% v/v) of formaldehyde used in the preparation of genomic DNA in the present study, isolates YO12 and YO15 did not yield clear DNA profiles following restriction and PFGE analysis (Fig. 2.2), lowering the typeability index to 92.8%. The concentration of DNases present in these two *F. johnsoniae*-like isolates is clearly problematic and further optimization of DNA preparation for PFGE is needed and recommended when typing *F. johnsoniae*-like isolates.

Clonal ancestors may give rise to genetically related groups of isolates with identical or similar phenotypes and/or physiological types, including biofilms phenotypes (Mack *et al*., 2000), if grown under the same conditions. Although PFGE types have been correlated with phenotypes and/or physiological types of

bacteria, such as biofilm formation (Borucki *et al*., 2003; Somers *et al*., 2001), variation in results obtained for different bacterial species exists. In *P. aeruginosa*, significant variation in biofilm phenotypes, motility, type IV pilus activity, growth rate and alginate production was observed amongst strains with identical genomic PFGE profiles (Head and Yu, 2004). In the present study it was not possible to correlate specific PFGE profiles with a specific biofilm phenotype. Interestingly, all the *F. johnsoniae*-like isolates displaying the strong biofilm phenotype, displayed high genetic relatedness in PFGE types and the specific WP3 WCP profile (Figs. 2.3 and 2.4). Statistically significant positive correlations were also observed between PFGE types and WCP and OMP types, respectively. Biofilm formation may be a strain-dependent characteristic of *F. johnsoniae*-like isolates and specific proteins, OMPs or other cell surface proteins, may be involved in the different biofilm processes.

Although it has been shown that motility, chitin degradation and proteolytic abilities of *F. johnsoniae*-like isolates and biofilm formation are opposing properties, the role of flavobacterial cellular proteins in biofilm formation is unknown. Specific protein components associated with the biofilm phenotype of *F. johnsoniae*-like isolates have not been identified. In order to identify specific proteins involved in biofilm formation, the following chapter will focus on the profiling of differentially expressed proteins in the planktonic, agar-surface-associated, and biofilm phases of growth using 2-D gel electrophoresis.

CHAPTER THREE

PROTEOMIC ANALYSIS OF *F. johnsoniae*-like PLANKTONIC, BIOFILM AND AGAR SURFACE-ASSOCIATED CELLS USING TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY.

3.1. INTRODUCTION

The biofilm mode of growth requires a change in bacterial physiology, which represents a fundamental change in bacterial gene expression. Generally, this physiological change involves the production of specific macromolecules essential for biofilm formation, such as proteins and polysaccharides, which are often associated with the cell surface, or more specifically the outer membrane. Different components, however, fulfill separate functions during the different phases of biofilm growth.

The series of events that take place during biofilm formation has been described as complex microbial developmental processes that influence bacterial physiology and give rise to unique phenotypes (O'Toole *et al*., 2000; Sauer *et al*., 2002). These regulated processes are very similar to the regulated swarming, sporulation and fruiting-body formation processes displayed by diverse aquatic and soil bacteria (O'Toole *et al*., 2000; Sauer *et al*., 2002). Hence, multiple phenotypes, based on structural and physiological related heterogeneity (Ghigo, 2003) may exist within a mature, multilayer biofilm, which complicates global expression studies (Beloin and Ghigo, 2005; Karatan and Watnick, 2009).

Although many proteomic and transcriptomic studies have delineated components of biofilm cells and certain phenotypic trends, including up-regulation of genes involved in EPS biosynthesis and genes involved in the adaptation to the stationary phase and stress conditions, there is no existing technique, or combination of techniques, that uniquely defines a specific biofilm fingerprint (Karatan and Watnick, 2009). Proteome analysis of differentially

expressed proteins over the course of biofilm development typically identify proteins involved in metabolic processes, such as amino acid metabolism, cofactor metabolism and other components involved in central and intermediary metabolism indicating metabolic shift and/or changes in carbon flux; EPS biosynthesis and excretion; membrane proteins, transporter proteins and components of motility structures; and proteins involved in adaptation and protection, including those associated with stress-response (Beyhan *et al*., 2007; Helloin *et al*., 2003; Jouenne *et al*., 2004; Karatan and Watnick, 2009; Prigent-Combaret *et al*., 1999; Sauer and Camper, 2001; Sauer *et al*., 2002; Seyer *et al*., 2005; Stanley and Lazazzera, 2004; Webb *et al*., 2003).

In *Peudomonas aeruginosa*, Sauer *et al.* (2002) demonstrated changes in regulation of many metabolic proteins, proteins involved in alginate production, several components of ABC transporters and general stress-response proteins during the five stages of biofilm development using the 2-D gel electrophoresis technique. Comparison of the planktonic proteome with that of the biofilm maturation-2 phase revealed 800 proteins that displayed six-fold or greater levels of expression in the biofilm phase (Sauer *et al*., 2002).

Vilain and Brözel (2006) demonstrated that the biofilm proteome of *Bacillus cereus* is distinct from planktonic cells in the exponential, transient and stationary phase using 2-D gel electrophoresis. Differential protein expression in terms of up- and down-regulation was responsible for the *B*. *cereus* biofilm phenotype rather than the expression of unique proteins (Vilain and Brözel, 2006).

Although the planktonic phase may not necessarily represent the preferred lifestyle of bacteria in their natural environment (Davies, 2000), it is the best studied phase of bacterial growth and used as the model phase for comparison of protein expression in the sessile phase of bacteria (Beloin and Ghigo, 2005). Only a small proportion of the planktonic proteome may display significant change in expression compared to that of a sessile mode of growth (Whiteley *et al*., 2001). However, certain up-regulated and/or uniquely expressed

proteins in the planktonic phase may hinder biofilm formation, such as in the case of TlpB in *Flavobacterium psychrophilum* (Álvarez *et al*., 2006).

The transcriptome and/or proteome of planktonic cells of *P. aeruginosa* (Hentzer *et al*., 2005; Seyer *et al*., 2005; Spoering and Lewis, 2001; Waite *et al*., 2005) and *B. cereus* (Vilain and Brözel, 2006) grown in the stationary phase have previously been compared to gene and protein expression in the biofilm phase and it has been suggested that biofilm cultures often display similarities to those of stationary phase planktonic cultures (Beloin and Ghigo, 2005). In *Escherichia coli*, biofilm and stationary phase planktonic cultures displayed similarity in stationary-phase-induced genes (Schembri *et al*. 2003). This may be explained by the suggested heterogeneous nature of stationary phase populations, comprising of cells that differ in growth status and gene expression patterns (Blokpoel *et al*., 2005).

Stress proteins may increase in the stationary phase of planktonic grown bacteria (Dukan and Nyström, 1998; Kikuchi *et al*., 2005) where cellular stress proteins, such as superoxide dismutase (SOD), may increase up to 100-fold in the stationary phase of Gram-negative bacteria (Kim *et al*., 2006). Typical proteins, including stress-related proteins, expressed in stationary phase planktonic cells, such as those associated with *Mycobacterium smegmatis* (Blokpoel *et al*., 2005), include heat shock proteins, electron transfer flavoproteins, DNA binding proteins, translational modification proteins and the translation elongation factor EF-Tu. Similar stress proteins may also be observed in the biofilm phase of bacteria (Helloin *et al*., 2003; Jouenne *et al*., 2004; Karatan and Watnick, 2009; Prigent-Combaret *et al*., 1999; Sauer and Camper, 2001; Sauer *et al*., 2002; Seyer *et al*., 2005).

Complex regulatory systems influence protein expression at the genetic level and play an important role in the regulation of differential gene expression between the planktonic and biofilm lifestyles (Beyhan *et al*., 2007; Ghigo, 2003; Stanley and Lazazzera, 2004). This produces specific biofilm phenotypes, each with unique proteomes, which may represent different stages of biofilm growth (Ghigo, 2003; Stanley and Lazazzera, 2004).

Components of regulatory systems, such as those involved in quorum sensing and two-component regulatory systems, are also frequently identified in biofilm proteomic studies (Helloin *et al*., 2003; Prigent-Combaret *et al*., 1999; Sauer *et al*., 2002). For instance, Sauer *et al*. (2002) demonstrated the up-regulation of several protein components involved in quorum sensing during the developmental stages of a *P. aeruginosa* biofilm. Signal transduction systems play a major role in the phenotypic changes that take place during *Pseudomonas* spp. biofilm formation (Sauer and Camper, 2001; Sauer *et al*., 2002). Using 2-D gel electrophoresis and SSH, Sauer and Camper (2001) demonstrated differential protein and gene expression took place in *P. putida* cells after attachment. Identified proteins that were up-regulated included motility proteins, transporter proteins, OMPs and proteins involved in polysaccharide biosynthesis (Sauer and Camper, 2001). Interestingly, down-regulated proteins also included motility proteins, transporter proteins and OMPs, as well as proteins involved in amino acid metabolism (Sauer and Camper, 2001). It was also demonstrated that cell-signaling mechanisms other than quorum sensing were involved in these changes (Sauer and Camper, 2001).

Critical questions which should be addressed prior to attempting a molecular biofilm study is whether the organism of interest possesses the ability to form biofilms and whether differential gene expression occurs during transition from the planktonic to the biofilm state by this organism. A basic approach for the latter involves protein profiling to investigate differences in the cellular proteome of planktonic and sessile cells, respectively (Vilain and Brözel, 2006). The *F. johnsoniae*-like isolates investigated in this study are known to form biofilms in flat-bed reactors and some of these isolates have also been cultured from biofilms present in aquaculture tanks (Basson *et al*., 2008; Flemming *et al*., 2007). However, differences in protein expression between the planktonic, biofilm and agar surface-associated phases of *F. johnsoniae*-like isolates have not been investigated previously. Pitta *et al*. (1993) observed differences in protein content of peptidoglycan for liquid-grown (planktonic) and surface-grown (agar surface-associated) cells of *F. johnsoniae* and suggested that this

organism was able to modify its cell envelope on surface contact. This is not surprising, since a unique gliding system is activated in *F. johnsoniae* when this organism comes into contact with solid surfaces and macromolecules present in the cell envelope, including transporter proteins and lipoproteins, are required for gliding motility (McBride, 2004). Moreover, modern proteomic and transcriptomic approaches have shown that planktonic and sessile forms of bacterial growth represent distinct physiological and metabolic states (Hentzer *et al.*, 2005; Rathsam *et al.*, 2005a and b; Ren *et al.*, 2004; Vilain and Brözel, 2006).

The mechanisms and cellular components involved in *Flavobacterium* spp. biofilm formation have not yet been elucidated. 2-D gel electrophoresis may reveal proteins involved in biofilm formation by *F. johnsoniae*-like isolates on abiotic surfaces and potentially those on biotic surfaces as well. Therefore, in the present study differences in protein expression between the planktonic, biofilm and agar surface-associated phases of WCP and OMP fractions of eleven *F. johnsoniae*-like isolates were investigated using 1-D SDS-PAGE. Two candidate isolates, YO12 and YO64, were then selected for further analysis using the 2-D gel electrophoresis technique to investigate differences in attachment between motile and non-motile *F. johnsoniae*-like isolates.

3.2. MATERIALS AND METHODS

3.2.1. Growth of *F. johnsoniae*-like Cultures

Eleven *F. johnsoniae*-like isolates previously assigned to specific phenotypes (colonial morphology, motility, proteolytic activity, and degree of adherence) (Basson *et al.*, 2008; Flemming *et al.*, 2007) and genotypes (RAPD-PCR, REP-PCR, WCP and OMP profiling) (Flemming *et al.*, 2007) were selected for WCP and OMP analysis of planktonic, biofilm and agar surface-associated growth, respectively (Table 3.1).

In order to harvest planktonic cells, 10 µl O/N cultures standardized to a 0.5

TABLE 3.1. Phenotypic, physiological and molecular characterization of the 11 *F. johnsoniae*-like isolates selected for preliminary proteomic analysis

Isolate	Degree of adherence* (26 °C)	WCP subtypes†	OMP subtypeS†	RAPD subtypes†	REP subtypes†	Colony type‡	Gliding motility†	Casein-proteolytic activity†	Chitinase activity (cm)	Extracellular carbohydrate content (ECC) § EAOA	
										OD_{480}	OD_{490}
YO12	W	W1	O1	R1	E2	H	+	+	2.5	2.96	2.67
YO15	W	W1	O2	R1	E2	H	+	+	2.43	7.62	6.91
YO19	S	W3	O3	R2	E3	S	-	(+)	-	2.01	1.82
YO34	S	W3	O4	R2	E5	S	-	(+)	-	1.76	1.43
YO45	M	W1	O5	R3	E6	H	+	+	2.07	2.50	2.20
YO51	W	W4	O7	R5	E8	S	-	+	1.97	1.74	1.59
YO53	W	W1	O9	R1	E2	H	+	+	2.17	3.23	2.82
YO59	S	W3	O12	R7	E10	S	-	+	-	0.57	0.54
YO60	W	W2	O11	R7	E10	H	(+)	+	2.33	2.18	1.93
YO64	S	W3	O14	R8	E11	S	-	(+)	-	0.90	0.75
YO66	M	W3	O16	R10	E13	S	-	(+)	-	1.41	1.24

* Degree of adherence in microtiter plate adherence assay - W,M and S denotes weak, moderate and strong adherence, respectively (Basson *et al.*, 2008).

† WCP, OMP, RAPD and REP profiles previously described by Flemming *et al.* (2007). For gliding motility, +, - and (+) denotes strong motility, nonmotile or weakly motile, respectively (Flemming *et al.*, 2007). For casein proteolytic activity, + and (+) denotes strong and weak casein proteolytic activity, respectively (Flemming *et al.*, 2007).

‡ H refers to hazy colony morphology and S refers to smooth colony morphology types (Flemming *et al.*, 2007).

§ Optical density values (480 nm and 490 nm) of the extracellular carbohydrate content (ECC) of isolates grown on Enriched Anacker and Ordal's medium (EAOA).

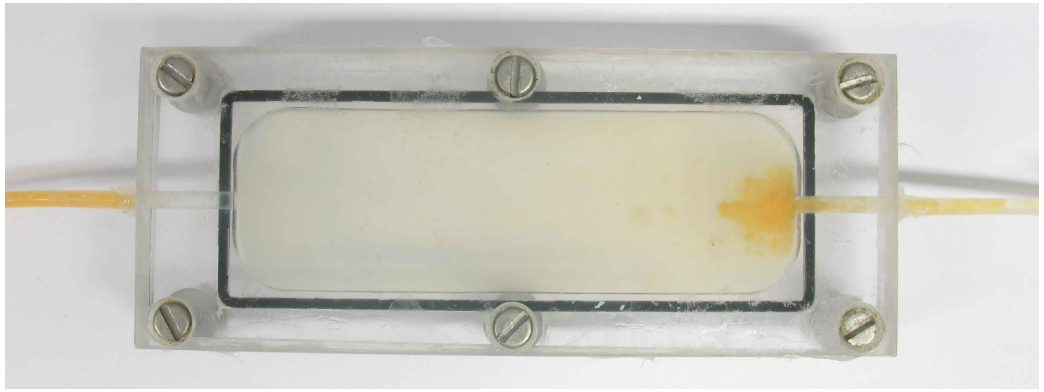

FIG. 3.1. Biofilm growth of isolate YO64 in a flat-plate reactor (channel size: 100 mm × 30 mm × 6 mm) of 36 h post-inoculation in enriched Anacker and Ordal's medium.

McFarland standard was used to inoculate 50 ml EAOB in flasks and incubated at RT on a shaker. A flow-through, flat plate Perspex flow cell reactor (channel size: 100 mm × 30 mm × 6 mm) (Fig. 3.1; Appendix 1), was used to harvest biofilm growth. Prior to inoculation, the flow cell with attached silicone tubing (1 × 1.6 mm × 3 mm × 5 m tubing; The silicone tube, RSA), attached with clear silicone sealant, were presterilized with liquid bleach for 1 h. The silicone tubing was connected, with the use of plastic adapters (T-connectors, 1/16"; straight connectors, 1/16" × 1/16"; Cole-Parmer Instrument Co., USA), to a reservoir containing 2 l of sterile EAOB. A peristaltic pump (Watson-Marlow, UK) was fitted on the silicone tubing, upstream of the flow cell. The flow cell was filled with EAOB and the flow rate was stabilized to 3 rpm (~260 ml/min) for 1 h. Clamps were used to seal silicone tubes upstream of each channel. Sterile syringes with needles were used to inoculate a volume of 1 ml pure overnight culture of each of the isolates YO12 and YO64, in respective flow-cells, below attached clamps. Flow cell systems were kept at RT throughout the experiment. Biofilm growth was harvested by shaking the flow cell system vigorously for 15 min after which the cells were centrifuged and resuspended in 10 ml PBS (pH 7). For agar surface-associated growth, O/N cultures standardized to a 0.5 McFarland standard were swabbed onto EAOA plates with sterile cotton-wool swabs and incubated at RT. Plate-grown cells were harvested using a wire loop and suspended in 10 ml PBS (pH 7).

All cultures (planktonic, biofilm and agar surface-associated) were grown for a 5 d period (120 h) before being harvested. Stationary phase, planktonic cells were harvested by centrifugation and resuspended in 10 ml PBS (pH 7).

3.1.1. WCP Profiles

Whole-cell proteins were prepared for planktonic, biofilm and agar surface-attached cells, respectively, of the 11 *F. johnsoniae*-like isolates (Table 3.1). Cultures were harvested as described above in section 3.2.1 and 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 of PBS buffer. Cell concentrations were standardized at OD_{595} to an absorbance of 1. Cells were sonicated using a Sonicator™ Cell Disruptor (Heat Systems-Ultrasonics Inc., USA) and 2 ml aliquots were stored at -20°C as WCP preparations. WCP isolations were carried out in triplicate for each respective isolate.

Protein preparations, in 50 µl volumes, 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, ddH_2O to 100 ml], heated for 5 min at 100°C and subjected to electrophoresis in 12% polyacrylamide gels by SDS-PAGE at 5 A for 14 h. A PageRuler™ prestained protein ladder (Fermentas, Canada) was used as protein marker. Proteins were stained with silver staining, using a modified procedure described by Tsai and Frasch (1982).

SDS-PAGE gels were immersed in fixing solution (50% methanol, 12% acetic acid, and 0.0185% formaldehyde) for 1 h. This was followed by 2 × 10 min washes in wash solution (50% ethanol). Thereafter, gels were pretreated in 0.02% sodium thiosulphate solution for 5 min and washed (3 × 30 s) in sterile ddH_2O. Gels were stained for 10 min in a silver nitrate solution (0.2% silver nitrate and 0.028% formaldehyde) and then washed (2 × 20 s) in sterile double-distilled water. Developer (6% sodium carbonate, 0.0185% formaldehyde, and 0.4 mg sodium thiosulphate) was added thereafter, and gels were agitated gently until protein bands appeared. Stop solution (50% methanol and 12% acetic acid) was added after sufficient development of protein bands.

WCP profiles were visually examined for differences in number, molecular weight and intensity of protein bands. Protein band sizes were calculated using UVIDOC V.97 (UVItec). WCP profiles of *F. johnsoniae*-like strains were compared with previously obtained WCP profiles (Flemming *et al.*, 2007). To ensure reproducibility, WCP profiles of protein preparations isolated in triplicate were compared.

3.1.2. OMP Profiles

OMPs for the 11 *F. johnsoniae*-like isolates were prepared with *N*-lauryl-sarcosine using a modification of the method described by Benedí and Martínez-Martínez (2001). *F. johnsoniae* cultures were harvested as described in section 3.2.1 and 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 of PBS buffer. Cell concentrations were standardized at OD_{595} to an absorbance of 1. Cells were sonicated using a Sonicator™ Cell Disruptor (Heat Systems-Ultrasonics Inc.) and centrifuged at 6000 rpm for 10 min. Supernatants were centrifuged at 37000 rpm for 1 h at 4°C in an L7-65 Ultracentrifuge (Beckman Instruments Inc, USA). Pellets were suspended in 2 ml of 2% sodium lauryl sarcosine (SLS) in PBS and incubated at room temperature for 30 min. Suspensions were centrifuged at 37 000 rpm for 45 min at 4°C. Resulting pellets were washed with 1% SLS in PBS and centrifuged at 37 000 rpm for 40 min at 4°C and subsequently used as partially purified OMP preparations. OMPs were resuspended in sterile ddH_2O and stored at -20°C. OMP isolations were carried out in triplicate for each respective isolate.

Protein preparations were solubilised in 20 µl 2 × sample buffer, heated for 5 min at 100°C and subjected to electrophoresis in 12% polyacrylamide gels by SDS-PAGE at 5 A for 14 h. A PageRuler™ prestained protein ladder (Fermentas) was used as protein marker. Proteins were stained with silver staining, using the modified procedure described by Tsai and Frasch (1982) as described previously in Section 3.2.2.

OMP profiles were visually examined for differences in number, molecular weight and intensity of protein bands. Protein band sizes were calculated using UVIDOC V.97 (UVItec). To ensure reproducibility, OMP profiles of protein preparations isolated in triplicate were compared.

3.1.3. 2-D Gel Electrophoresis

Two isolates, YO12 and YO64, were selected for 2-D gel electrophoresis based on their biofilm-forming ability, producing weak and strong biofilm phenotypes, respectively (Table 3.1). Isolate YO12 was weakly adherent in microtiter adherence assays, but displayed strong motility, casein proteolytic and chitinase abilities and a hazy colonial morphology. In contrast to isolate YO12, YO64 was strongly adherent, displayed no motility or chitinase activity, weak casein proteolytic activity, and a smooth colonial morphology. WCP and OMP preparations of isolates YO12 and YO64 were prepared as described in sections 3.2.2 and 3.2.3 and subjected to 2-D gel electrophoresis.

Three hundred micrograms of WCP from each growth phase were mixed with 0.8% (v/v) DTT, 0.2% (v/v) ampholytes (Bio-Rad), 0.001% bromophenol blue and made up to a final volume of 125 µl using urea buffer (9 M urea, 2 M thiourea and 4% CHAPS). Samples were then placed in individual channels of an Immobiline™ Dry Strip Reswelling Tray (GE Healthcare, Amersham, UK). Linear, 7 cm ReadyStrip™ IPG strips pH range 4-7 (Bio-Rad) were carefully placed on top of the samples, gel side being directly in contact with the sample, avoiding trapping any air bubbles in the process. The strips were then covered with mineral oil (PlusOne DryStrip Cover Fluid; GE Healthcare) and left to passively rehydrate to their original gel thickness of 0.5 mm for at least 15 h at RT.

After rehydration, IPG strips were briefly rinsed with ddH_2O and carefully blotted with moist filter paper. The strips were placed gel side up on the focusing platform of an Ettan™ IPGphor II™ (GE Healthcare). Wicks moistened in ddH_2O were placed at the extreme ends of both the anodic and cathodic ends of the IPG strips to collect excess salts and impurities from the sample during focusing. The IPG strips were covered with mineral oil and isoelectric focusing was performed in a three phase stepwise programme: 250 V for 15 min, 4000 V for 1 h, and 4000 V for 12000 V-h at 20°C.

After IEF, IPG strips were equilibrated in SDS-containing buffers prior to second dimension SDS-PAGE. The focused IPG strips were incubated gel side up in reswelling tray channels containing 2.5 ml equilibration buffer [6 M urea, 2% (w/v) SDS, 50 mM Tris-Cl, pH 8.8 and 20% (v/v) glycerol], firstly containing 2% (w/v) DTT for 15 min followed by 2.5% (w/v) iodoacetamide for another 15 min with gentle agitation at room temperature. After equilibration, the isoelectric focused proteins were ready for separation by second-dimension SDS-PAGE.

Mini format 2-D SDS-PAGE gels were cast on 10.1 cm (width) x 8.3 cm (height) spacer glass plates (Bio-Rad) mounted with 1 mm thick spacers using the Mini-PROTEAN→3 Multi-Casting Chamber (Bio-Rad). Equilibrated 7 cm IPG strips were gently rinsed with 1× SDS-PAGE running buffer and placed on top of mini format 12% SDS-PAGE resolving gels with the plastic backing against the spacer plate. Three microlitres of PageRuler™ unstained protein ladder (Fermentas) were spotted on small pieces of filter paper, air-dried and placed on the SDS-PAGE gel. The IPG strips were then overlaid with 1 ml of 0.5% (w/v) molten agarose prepared in 1× SDS-PAGE running buffer containing a tint of bromophenol blue, which was used as a migration tracking dye during electrophoresis. Electrophoresis was carried out using the Mini-PROTEAN→3 Dodeca™ cell (Bio-Rad) at 100 V during the first 30 min and then at 150 V until the bromophenol dye reached the bottom of the glass plates.

OMP samples were initially analyzed using 2-D gel electrophoresis, however, these samples proved to be highly hydrophobic and all attempts to remove streaks and increase the resolution failed. These samples were then separated using 1-D SDS-PAGE (section 3.2.3).

Proteins separated by 2-D SDS-PAGE were detected using a modified Coomassie brilliant blue (CBB) R-250 staining protocol with three sequential staining steps. After electrophoresis, gels were immersed firstly in CBB staining solution I [0.025% (w/v) CBB R-250, 10% (v/v) glacial acetic acid and 25% (v/v) propan-2-ol], heated for 1 min in a microwave at maximum power and incubated for 30 min with shaking at room temperature. The CBB staining solution I was discarded and the staining process was repeated using CBB staining solution II

[0.003% (w/v) CBB R-250, 10% (v/v) glacial acetic acid and 10% (v/v) propan-2-ol] and finally CBB staining solution III [0.003% (w/v) CBB R-250 and 10% (v/v) glacial acetic acid] for 30 min in each stain as described above. After staining, gels were immersed in destaining solution [10% (v/v) acetic acid and 1% (v/v) glycerol] with shaking at RT until the protein spots were visibly distinct against a clear background. Gels were imaged using a Molecular Imager PharosFX Plus System (Bio-Rad).

WCP and OMP samples were prepared and separated electrophoretically in triplicate for each isolate to evaluate reproducibility. Qualitative and quantitative analysis of the 2-D protein spot patterns was carried out using PDQuest software Version 6.21 (Bio-Rad). Standardized gels were compared for differential expression analysis.

A selection of differentially-expressed WCP spots and OMP bands, as well as WCP spots and OMP bands expressed uniquely in each of the planktonic, biofilm and agar surface-associated phases of isolates YO12 and YO64, respectively, were selected for MALDI-TOF MS identification.

3.1.4. Protein Identification with MALDI-TOF MS

Protein spots/bands of interest were excised manually from 2-D and 1-D gels and transferred into sterile microcentrifuge tubes. Gel pieces were washed twice with 500 µl of 50 mM ammonium bicarbonate for 5 min each time and a third time for 30 min, vortexing occasionally. Gel pieces were destained twice with 500 µl of 50% (v/v) 50 mM ammonium bicarbonate and 50% (v/v) acetonitrile for 30 min, vortexing occasionally. These were dehydrated with 100 µl of 100% (v/v) acetonitrile for 5 min, and completely dessicated using the Speed Vac SC100 (ThermoSavant, Waltham, MA, USA). Proteins were digested in-gel with approximately 120 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) dissolved in 25 mM ammonium bicarbonate, for 6 h at 37°C. Protein

digestion was stopped by adding 50 µl of 1% (v/v) TFA and incubating 2 h at room temperature before storage at 4°C until further analysis.

Prior to identification, samples were cleaned-up by reverse phase chromatography using ZipTip $_{C18}$™ (Millipore, Billerica, MA, USA) pre-equilibrated first in 100% (v/v) acetonitrile and then in 0.1% (v/v) TFA and were eluted out with 50% (v/v) acetonitrile. Digested proteins (1 µl) were mixed separately with the same volume of α-cyano-4-hydroxycinnamic acid (CHCA) matrix and spotted onto a MALDI target plate for analysis by MALDI-TOF MS using a Voyager DE Pro Biospectrometry workstation (Applied Biosystems, Forster City, CA, USA) to generate peptide mass fingerprints (PMFs). The MALDI-TOF MS was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential with optimized parameters. Close external calibration was employed using the Sequazyme calibration™ mixture II containing angiotensin I, ACTH (1-17 clip), ACTH (18-39 clip) and bovine insulin (Applied Biosystems). This calibration method typically provided mass accuracy of 200 ppm across the mass range 900 to 5,000 Da. Peptide spectra of accumulated 1,200 shots each were automatically processed for baseline correction, noise removal, and peak deisotoping. Threshold was manually adjusted between 2 and 8% base peak intensity. Proteins were identified by comparing the generated MALDI-TOF mass spectra against the NCBInr, MSDB and Swiss-Prot peptide mass databases using the MASCOT algorithm (http://www.matrixscience.com/search_form select.html).

Probability-based Mowse scores [protein score was -10*Log(P), where P is the probability that the observed match is a random event] were calculated and putative functions were assigned to the selected proteins based on significant matches to known proteins ($p < 0.05$). Candidate identifications with MOWSE scores higher than 85 were automatically considered as positive assignments. All other assignments with MOWSE score greater than 64 were considered positive if more than 10% of protein sequence was covered. If more than one

protein satisfied mentioned threshold criteria, the entry with the highest MOWSE score was assigned.

3.2. RESULTS

3.2.1. WCP Analysis

Variability was observed in the WCP profiles of planktonic, biofilm and agar surface-associated cells of the *F. johnsoniae*-like isolates (Fig. 3.2). WCP profiles and protein molecular weights of four *F. johnsoniae*-like isolates (YO12, YO19, YO45 and YO64) tested in each of the three different growth phases, viz., planktonic, biofilm and agar surface-associated phases, respectively, are listed in Table 3.2. Although WCP profiles of planktonic, biofilm and agar surface-associated phases displayed an overall similarity for each respective isolate, distinct differences in presence/absence of protein bands were observed (Fig. 3.2; Table 3.2; and Tables 2A - C, Appendix 2).

WCP profiles of the biofilm phase displayed the highest percentage uniquely-expressed proteins. In comparison to planktonic WCP profiles, those of biofilm cells displayed the lowest similarity. Approximately 73% of the isolates contained more bands in biofilm WCP profiles compared to their planktonic WCP profiles (except for isolates YO12, YO45 and YO64). The WCP profile of isolate YO19 biofilm cells displayed the most protein bands (41) and the highest number of uniquely-expressed proteins (23) when compared to the planktonic phase (Table 3.2). Prominent proteins (present in WCP profiles of more than 30% isolates) expressed in the biofilm phase, but not in the planktonic phase included WCP bands of 146.5 kDa, 119 kDa, 81.4 kDa, 80.6 kDa, 74.5 kDa, 70 kDa, 66.5 kDa, 54 kDa, 52 kDa, 51 kDa, 49 kDa, 45.5 kDa, 41.5 kDa, 32.5 kDa, 29.5 kDa, 29.3 kDa, 27.5 kDa, and 26 kDa. Many protein band differences between WCP profiles of planktonic and biofilm grown cells were observed in the 100 - 179.3 kDa range and 25 - 35 kDa range. Approximately 91% of the isolates displayed

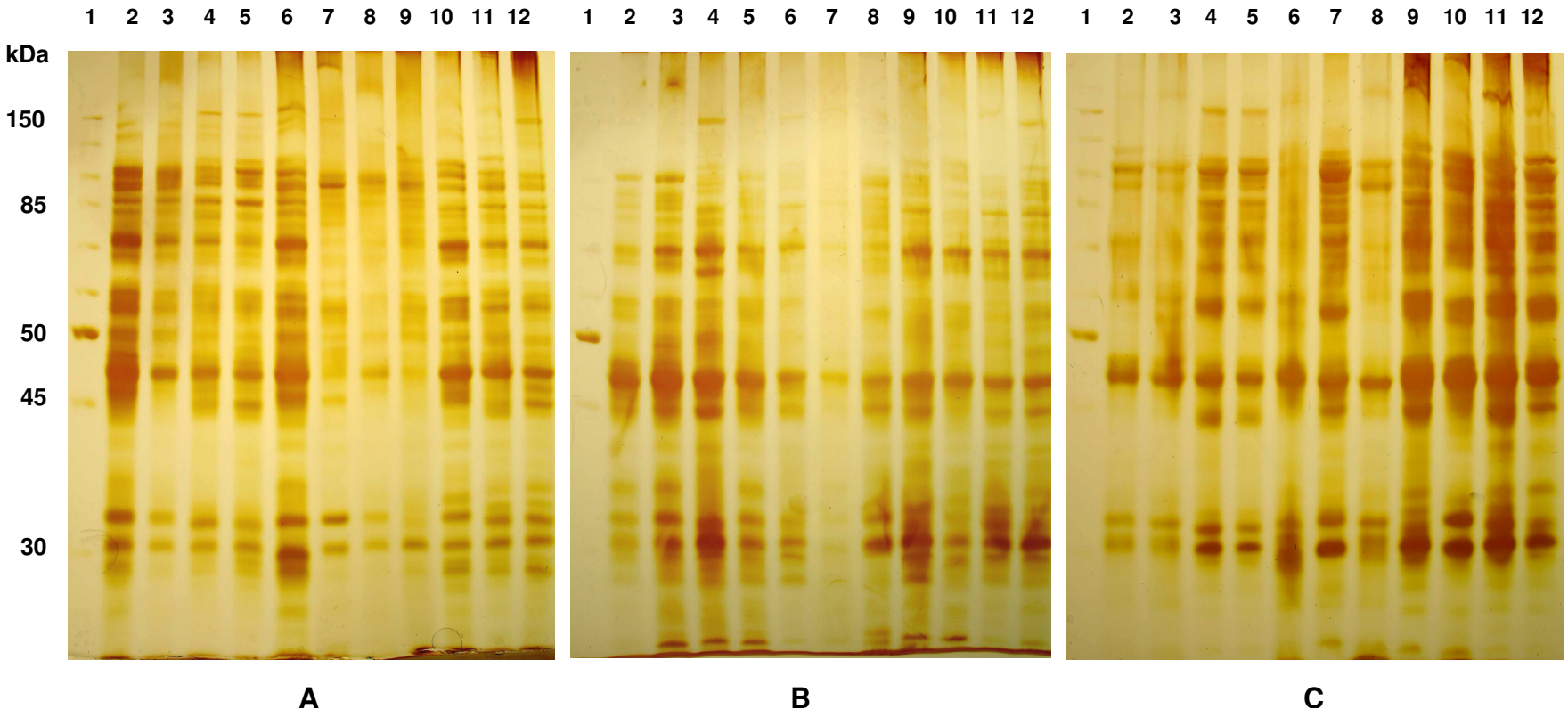

FIG. 3.2. Electrophoregrams displaying whole cell protein profiles of planktonic (A), biofilm (B), and agar surface-associated (C) *F. johnsoniae*-like isolates. Lanes A1, B1 and C1: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/25/20/15/10 kDa) (Fermentas, Canada); lanes A2-A12, B2-B12, and C2-C12: isolates YO12, YO15, YO19, YO34, YO45, YO51, YO53, YO59, YO60, YO64, and YO66, respectively.

TABLE 3.2. Whole cell protein profiles of planktonic (P), biofilm (B) and agar surface-associated (AS) cells of *F. johnsoniae*-like isolates YO12, YO19, YO45 and YO64

Isolate	YO12			YO19			YO45			YO64		
Growth Phase	P	B	AS	P	B	AS	P	B	AS	P	B	AS
No. of bands	30	26	20	30	41	27	30	28	21	34	31	31
No. of unique bands	11	12	3	6	23	10	11	13	5	15	16	11
Sizes (kDa)	179.3		179.3				179.3	179.3	179.3	179.3	179.3	179.3
									159			
				150		150						
					146.5			146.5				
	145			145		145	145			145		
								143				
					134.5							
	126			126	126	126	126			126		126
					119							
			113			113			113			113
											111	
										110		
												107
											106	
				105	105	105	105	105		105		
	102	102	102					102	102			
				101	101	101	101			101	101	101
				100						100		
					97			96		96	96	
				96	96						95	
						95						
	93	93	93		93		93		93			93
	86	86	86	86		86	86	86	86	86		86
											84.5	
	84				84						84	
								83				
	82	82					82					
					81.4						81.4	
					80.6						80.6	
				80		80		80		80		80
					79							
	77						77					
					76							
	73.0		73.0	73	73	73	73			73	73	
		70.0			70			70			70	70
				69	69			69		69		
	68	68	68			68	68		68			68
				67.3						67.3		
								67				
					66.5						66.5	
						66						66
				64						64		
											63	
	62		62				62		62			
		61		61	61					61		
	60	60	60	60	60		60	60	60		60	60
		59			59					59		
						57					58.5	
												57
	56	56	56	56	56		56	56	56			
											55	
					54							
										53.7		
					52			52			52	52
		51										
	50			50	50	50	50	50		50		50
		49									49	
					47		47			47		
	46		46	46		46	46		46			46
		45.5								45		
	44	44	44	44	44	44	44	44	44	44	44	44
	42			42						42		42
		41.5			41.5							
	41						41					
			40	40				40	40	40		40
						39.5						
		39.3		39.3	39.3			39.3		39.3	39.3	39.3
	39						39					
	37	37	37	37	37	37	37	37		37	37	37
										36.5		
				36	36						36	36
	35	35				35	35	35				35
	34.7						34.7					34
			34.5						34.5		34.5	
	34					34	34					
										33.6		
	33.5		33.5				33.5		33.5		33.5	
		33		33	33	33		33		33		33
					32.5						32.5	
	32		32				32		32			
				31.7		31.7				31.7		31.7
									31.5			
	31	31	31	31	31	31	31		31	31	31	31
								30.5				
				30		30			30	30		30
		29.5			29.5			29.5			29.5	
					29.3							
	29	29		29	29		29	29		29	29	
						28.5						28.5
		28		28						28		
					27.5							
	27	27	27		27		27	27	27	27	27	
						26.7						26.7
					26.5							
		26						26				
	25	25	25		25	25	25	25	25		25	25

more bands in biofilm WCP profiles compared to their agar surface-associated WCP profiles (except isolate YO64, which displayed an equal number of protein bands for each of these phases). Prominent proteins expressed in the biofilm phase, but not in the agar surface-associated phase included WCP bands of 146.5 kDa, 119 kDa, 97 kDa, 96 kDa, 81.4 kDa, 80.6 kDa, 69 kDa, 66.5 kDa, 63 kDa, 61 kDa, 59 kDa, 54 kDa, 51 kDa, 45.5 kDa, 41.5 kDa, 32.5 kDa, 29.5 kDa, 29.3 kDa, 29 kDa, and 28 kDa. The WCP bands of 29.5 kDa and 29 kDa were present in 90% to 100% of WCP profiles in the biofilm phase, respectively. Prominent biofilm WCP bands of 70 kDa (present in 100% of WCP profiles in the biofilm phase), 67 kDa, 52 kDa and 26 kDa, absent in the planktonic phase, were also present in WCP profiles of isolates in the agar surface-associated phase.

WCP profiles of agar surface-associated cells displayed the least uniquely expressed proteins. In comparison to planktonic WCP profiles, those of agar surface-associated cells displayed the highest similarity. Prominent proteins uniquely expressed in the agar surface-associated phase, but not in the planktonic phase included WCP bands of 113 kDa, 66 kDa, 57 kDa, 28.5 kDa, and 26.7 kDa. Prominent proteins expressed in the agar surface-associated phase, but not in the biofilm phase included WCP bands of 145 kDa, 113 kDa, 66 kDa, 62 kDa, 57 kDa, 42 kDa, 32 kDa, 31.7 kDa, 30 kDa, 28.5 kDa, and 26.7 kDa. A WCP band of 70 kDa was the only prominent agar surface-associated WCP band present in the biofilm phase, but absent in the planktonic phase.

3.1.1. OMP Analysis

OMP profiles of planktonic, biofilm and agar surface-associated cells also displayed a large degree of variability and many differences in presence/absence of protein bands were observed (Fig. 3.3). OMP profiles and protein molecular weights of four *F. johnsoniae*-like isolates (YO12, YO19, YO45 and YO64) tested in each of the 3 different growth phases, viz., planktonic, biofilm and agar surface-associated phases, respectively, are listed in Table 3.3.

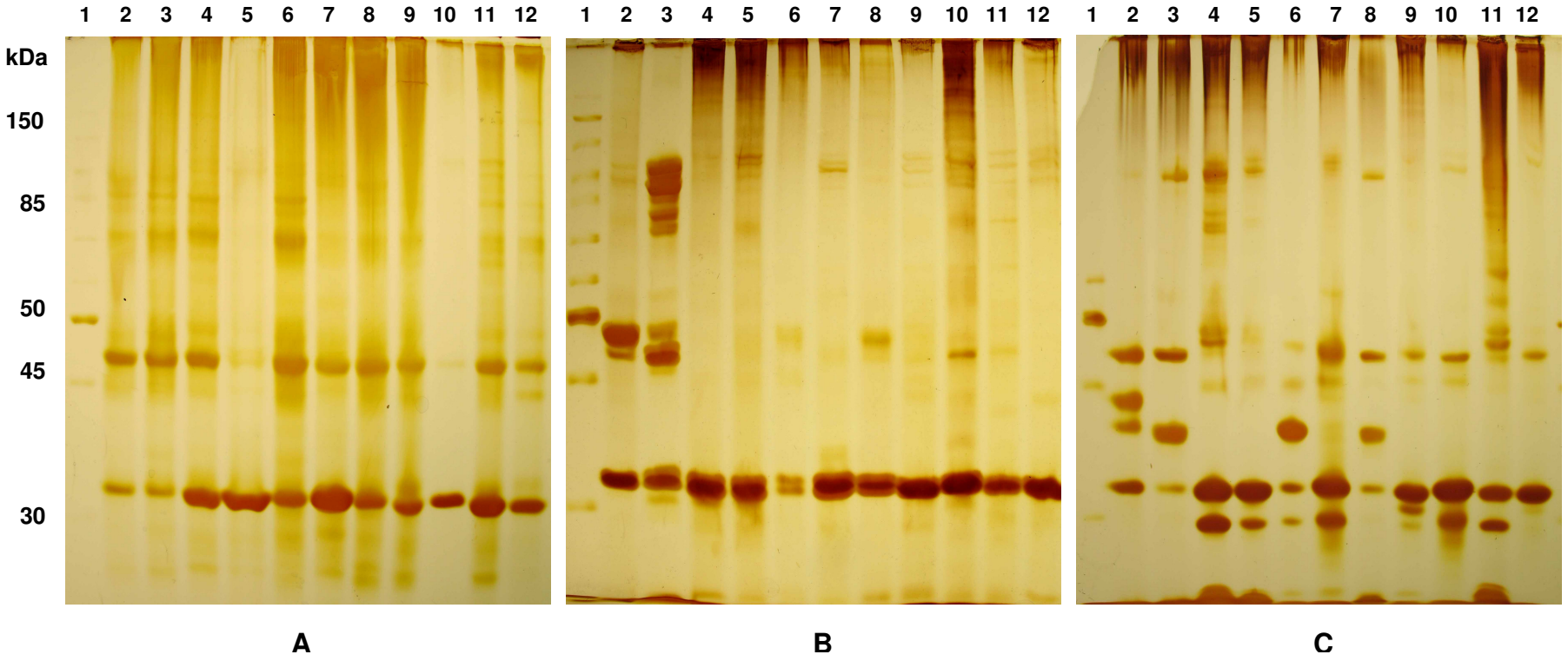

FIG. 3.3. Electrophoregrams displaying outer membrane protein profiles of planktonic (A), biofilm (B), and agar surface-associated (C) *F. johnsoniae*-like isolates. Lane 1: PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/25/20/15/10 kDa) (Fermentas, Canada); lanes A2-A12, B2-B12, and C2-C12: isolates YO12, YO15, YO19, YO34, YO45, YO51, YO53, YO59, YO60, YO64, and YO66, respectively.

TABLE 3.3. Outer membrane protein profiles of planktonic (P), biofilm (B) and agar surface-associated (AS) cells of *F. johnsoniae*-like isolates YO12, YO19, YO45 and YO64

Isolate	YO12			YO19			YO45			YO64		
Growth phase	P	B	AS	P	B	AS	P	B	AS	P	B	AS
No. of bands	18	13	8	18	17	23	20	11	9	23	20	22
No. of unique bands	15	11	5	9	9	14	17	8	5	16	10	14
Sizes (kDa)		230				220						
					200							
								190			190	
						180						180
					175							
											170	
						165						165
		160			160							
	150						150			150		
				149	149			149			149	
	135									135		
				130	130		130					
						129						
					125							
										120		
					115							
											112	112
			110		110							
										109		
						108			108			108
		106			106			106			106	
			105			105						105
			104	104								
							103			103		
	101											
		100		100	100						100	100
							99					
	95					95						
											94	
				92						92		
	90				90	90		90				
		87									87	
	86											
				85		85	85			85	85	85
	80			80		80	80			80	80	
		78										79
											76	76
						75						75
				73		73				73		
	72											72
							70			70		
		69										69
	68					68						
												67
										66		
				65		65	65					
												64
										63	63	
				62								
						57				57		
			56						56			56
		55				55	55	55				
										54		
							50					
								49		49	49	
		48		48		48		48				48
	47				47	47	47				47	47
									46			
						45.5						
	45	45	45	45			45		45	45	45	45
										42		
					41							
		40			40	40		40	40			40
	39.5			39.5			39.5			39.5		
			39									
	37		37								37	
							36.7			36.7		
		36							36			
				35.5				42				
	35						35					
	33.5	33.5	33.5				33.5	33.5	33.5			
				33	33	33		33		33	33	33
					32							
						30			30			
	29.9						29.9			29.9		29.9
											29.5	
				29	29		29			29		
				28.5								
	28						28				28	
				27.5						27.5		
	27.3						27.3					
												27
						26			26		26	

Majority of differences observed in OMP profiles from all three growth phases were in the 69 - 220 kDa region (Tables 2D - F, Appendix 2). Ten OMPs unique to the biofilm and agar surface-associated phases (220 kDa, 200 kDa, 180 kDa, 170 kDa, 112 kDa, 106 kDa, 105 kDa, 76 kDa, and 69 kDa) were observed in this region. Over-expressed proteins present in OMP profiles of the planktonic phase, but absent or repressed in the biofilm phase included an OMP band of 45 kDa (Fig. 3.3A).

Prominent proteins (present in OMP profiles of more than 30% isolates) expressed in the biofilm phase, but not in the planktonic phase included OMP bands of 190 kDa, 175 kDa, 106 kDa, and 32 kDa. Over-expressed proteins present in OMP profiles of the biofilm phase, but absent or repressed in the planktonic phase included an OMP band of 48 kDa (Fig. 3.3B). Prominent proteins expressed in the biofilm phase, but not in the agar surface-associated included OMP bands of 190 kDa, 175 kDa, and 49 kDa.

Prominent proteins expressed in the agar surface-associated phase, but not in the planktonic phase included OMP bands of 220 kDa, 180 kDa, 165 kDa, 112 kDa, 108 kDa, 105 kDa, 75 kDa, and 39 kDa. Over-expressed proteins present in OMP profiles of the agar surface-associated phase, but absent or repressed in the planktonic or biofilm phases included an OMP band of 30 kDa (Fig. 3.3C). Prominent proteins expressed in the agar surface-associated phase, but not in the biofilm phase included OMP bands of 165 kDa, 108 kDa, 75 kDa, 56 kDa, 39 kDa, and 30 kDa. Abundant proteins expressed in the biofilm and agar surface-associated phases, but not in the planktonic phase, included OMP bands of 220 kDa, 180 kDa, 112 kDa, 106 kDa, and 105 kDa.

3.1.1. 2-D Gel Electrophoresis and MS

Although both YO12 and YO64 were identified as *F. johnsoniae*-like isolates by 16S rRNA gene sequence analysis, the proteomes of these two isolates were clearly distinct (Figs. 3.4 and 3.5). While planktonic, biofilm and agar surface-

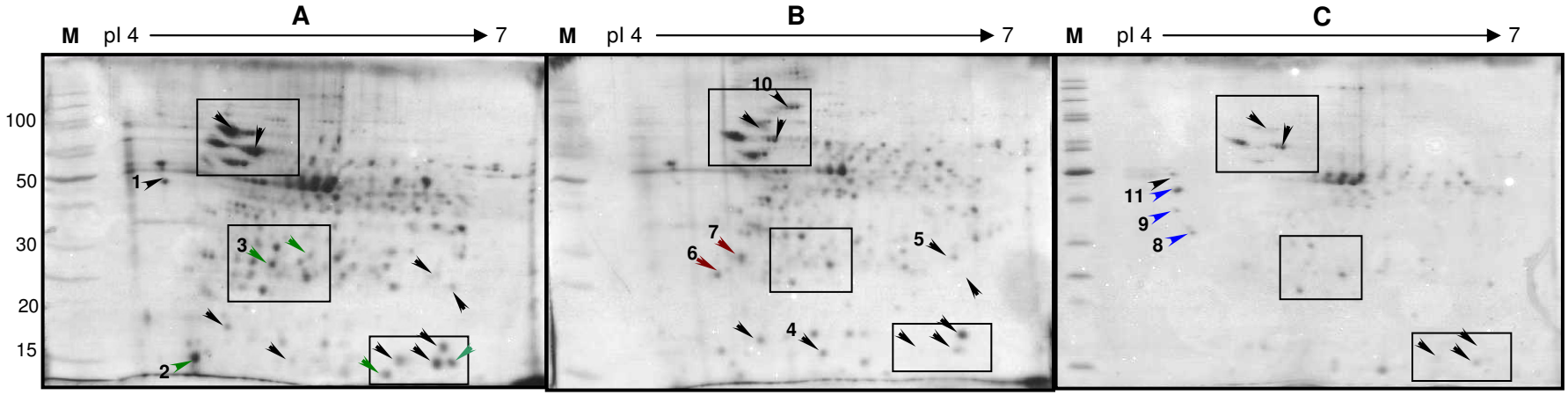

FIG. 3.4. 2-D gel electrophoresis WCP spot profiles of isolate YO12 grown in the planktonic (A), biofilm (B), and agar surface-associated (C) phases, respectively. PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/25/20/15/10 kDa; Fermentas, Canada) was used as protein marker (M). Horizontal axes represent pI values of isoelectric focusing gradients. Arrows numbered 1-11 represent identified proteins. Black arrows indicate differentially expressed proteins and coloured arrows indicate uniquely expressed proteins in each respective growth phase. Examples of differentially expressed clusters of proteins are boxed.

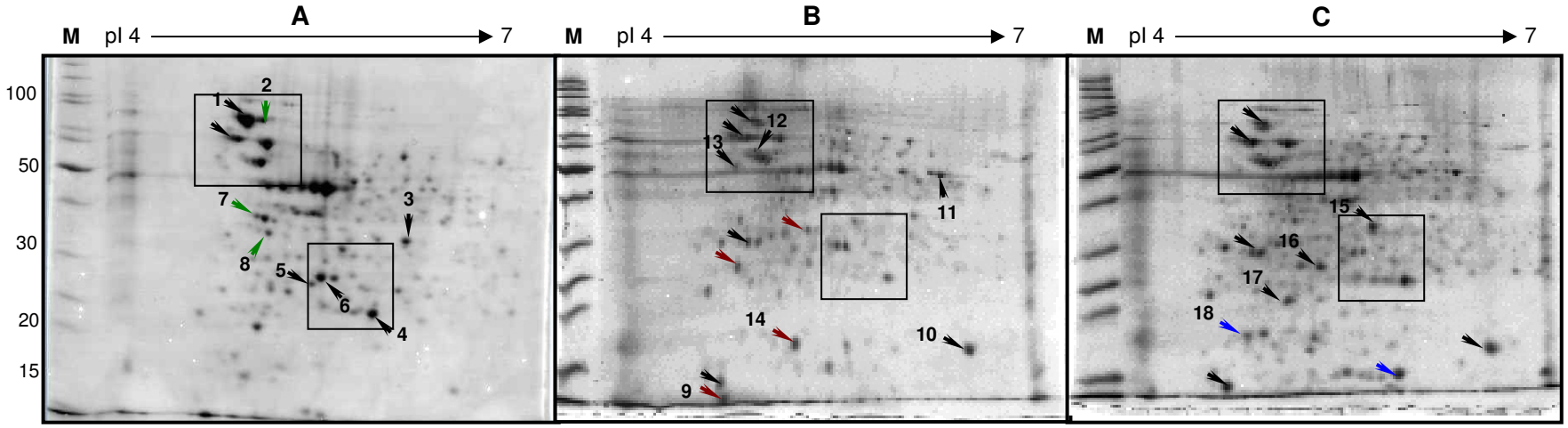

FIG. 3.5. 2-D gel electrophoresis WCP spot profiles of isolate YO64 grown in the planktonic (A), biofilm (B), and agar surface-associated (C) phases, respectively. PageRuler™ unstained protein ladder (200/150/120/100/85/70/60/50/40/30/25/20/15/10 kDa; Fermentas, Canada) was used as protein marker (M). Horizontal axes represent pI values of isoelectric focusing gradients. Arrows numbered 1-18 represent identified proteins. Black arrows indicate differentially expressed proteins and coloured arrows indicate uniquely expressed proteins in each respective growth phase. Examples of differentially expressed clusters of proteins are boxed.

associated phase 2-D gels of isolates YO12 and YO64, respectively, displayed an overall similarity in WCP spot patterns over the pH 4 - 7 range, distinct differences in presence/absence of spots, as well as variation in expression were observed (Figs. 3.4 and 3.5). Analysis of the generated 2-D electrophoregrams revealed over-expressed and/or repressed proteins and growth phase-specific proteins present in each of planktonic, biofilm and agar surface-associated samples examined, respectively (Figs 3.4 and 3.5). Distinct protein clusters where proteins presented as a series of differentially expressed spots are boxed (Figs. 3.4 and 3.5).

Planktonic, biofilm and agar surface-associated phase 1-D gels displayed a large degree of variation in OMP band patterns. OMP profiles also revealed over-expressed and/or repressed proteins and growth phase-specific proteins present in each of planktonic, biofilm and agar surface-associated samples examined, respectively (Figs 3.6 and 3.7).

Sixteen proteins from isolate YO12 were selected for MS analysis (Figs 3.4 and 3.6, Table 3.4) and were characterized according to function. These represented several different categories, viz., metabolic processes, transcription/translation, adaptation/protection, membrane proteins/transport/ motility, and hypothetical function (Table 3.5).

Seventy-five percent of isolate YO12 protein spectra displayed homology to previously characterized *Flavobacterium* spp. proteins (Tables 3.4 and 3.5). Of these, only 41.7% displayed significant homology to *F. johnsoniae* UW101 proteins (Table 3.4). While protein spot YO12_11 from the agar surface-associated phase could not be identified, the remaining 15 were assigned to four functional categories (Table 3.5). Of these, four proteins were hypothetical proteins of unknown function. Protein spots unique to the planktonic phase included a putative transaldolase C (YO12_3) involved in metabolic processes and proteins with unknown function (YO12_2 and YO12_12) (Table 3.5). In the biofilm phase, a putative alkyl hydroperoxide reductase (YO12_7) involved in adaptation/protection was uniquely expressed in addition to proteins associated with metabolic processes (YO12_6), transcription/translation (YO12_14), and

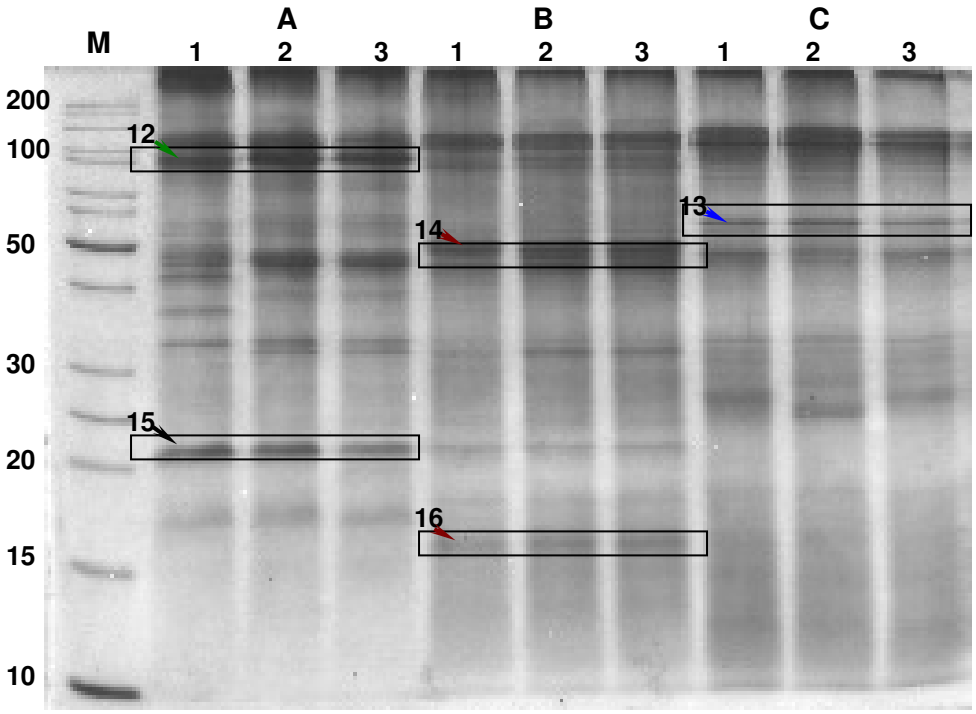

FIG. 3.6. Electrophoregram displaying OMP profiles of planktonic (A 1-3), biofilm (B 1-3), and agar surface-associated (C 1-3) cells of isolate YO12 separated by 1-D gel electrophoresis. Protein samples were prepared and run in triplicate for each respective growth phase. Arrows 12-16 represent identified proteins. Lane M: PageRuler™ unstained protein Ladder (200/150/120/100/85/70/60/50/40/30/25/20/15/10 kDa; Fermentas, Canada).

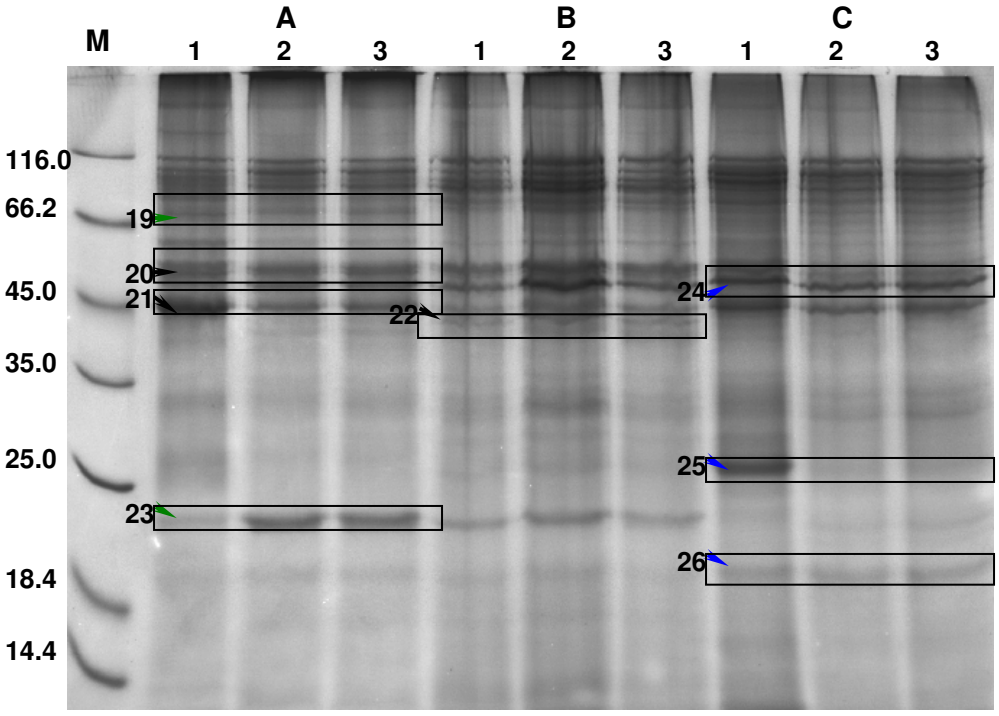

FIG. 3.7. Electrophoregram displaying OMP profiles of planktonic (A 1-3), biofilm (B 1-3), and agar surface-associated (C 1-3) cells of isolate YO64 separated by 1-D gel electrophoresis. Protein samples were prepared and run in triplicate for each respective growth phase. Arrows 19-26 represent identified proteins. Lane M: unstained protein molecular weight marker (116/66.2/45/35/25/18.4/14.4;Fermentas,Canada).

TABLE 3.4. Identification of WCP and OMP spots/bands of planktonic, biofilm and agar surface-associated phases of *F. johnsoniae*-like isolate YO12

Protein no.	Growth phase	Identified Protein (Gene name)*	Mass (kDa)	pI	Organism	Sequence Coverage (%)	Mowse Score	Significance (Mowse score)	Accession no.	No. of Matched Peptides
YO12_1	Planktonic (WCP)	OmpA/MotB domain protein (*ompA*; Fjoh_0697)	51.25	4.88	*Flavobacterium johnsoniae*	17%	68	>66	Q1XN88	3
YO12_2	Planktonic (WCP)	Hypothetical protein FB2170_14393 (FB2170_14393)	23.99	4.6	Flavobacteriales bacterium	28%	72	>70	gi|88712086	11
YO12_3	Planktonic (WCP)	Transaldolase C (*talC*; Fjoh_4567)	23.55	5.02	*Flavobacterium johnsoniae*	36%	69	>66	Q1XQA9	4
YO12_12	Planktonic (OMP)	Hypothetical protein Plut_1814 (Plut_1814)	14.65	9.98	*Pelodictyon luteolum*	54%	71	>66	Q3B1W3	2
YO12_15	Planktonic (OMP)	Hypothetical protein Fjoh_3665 (Fjoh_3665)	41.81	6.31	*Flavobacterium johnsoniae*	22%	75	>70	Gi 146301407	6
YO12_4	Biofilm (WCP)	Uridylate kinase (*pyrH*)	25.49	6.62	*Prochlorococcus marinus*	28%	58	>57	PYRH	2
YO12_5	Biofilm (WCP)	Conserved hypothetical protein (DR2079)	15.28	9.13	*Deinococcus radiodurans*	47%	76	>66	H75316	2
		MutT/nudix family protein (DR1025)	17.56	5.01	*Deinococcus radiodurans*	30%	64	>66	C75446	
YO12_6	Biofilm (WCP)	Inorganic diphosphatase (*ppa*; Fjoh_1478)	20.23	4.56	*Flavobacterium johnsoniae*	22%	78	>66	Q1XVR3	2
YO12_7	Biofilm (WCP)	Alkyl hydroperoxide reductase/thiol specific antioxidant (*ahpC*; Fjoh_2117)	23.58	4.67	*Flavobacterium johnsoniae*	26%	84	>66	Q1XSS4	18
YO12_10	Biofilm (WCP)	Translation elongation factor G (*fusA;* Fjoh_0399)	79.13	5.05	*Flavobacterium johnsoniae*	27%	78	>66	Q1XQN7	4
YO12_14	Biofilm (OMP)	Putative thiol, disulfide interchange protein (*dsbA*)	44.97	5.83	Flavobacteria bacterium	29%	70	>66	Q26DL2	4
YO12_16	Biofilm (OMP)	Nitrogenase iron protein/ABC transporter (*nifH*)	13.31	4.23	Nitrogen-fixing bacterium	46%	84	>70	Gi 28864305	4
YO12_8	Agar (WCP)	Catalase (Peroxidase I) (*katA*)	82.91	4.89	Flavobacteria bacterium	16%	68	>66	Q26FG8	2
YO12_9	Agar (WCP)	Spo0B-related GTP binding protein (*obgE*)	36.71	5.55	Flavobacteriales bacterium	37%	72	>70	Gi 163786228	4
YO12_11	Agar (WCP)	Not Identifiable								
YO12_13	Agar (OMP)	Glycerol kinase (*glpK*)	119.09	6.19	*Flavobacterium* spp.	12%	78	>70	Gi 86140681	3

* Gene name corresponding to identified protein indicated within ().

TABLE 3.5. Differential expression of identified WCP spots and OMP bands in the planktonic, biofilm and agar surface-associated phases of *F. johnsoniae*-like isolate YO12

Growth Phase	Protein Expression	Protein Spot	Protein ID	Functional Annotation
Planktonic	Up-regulated	YO12_1	OmpA/MotB domain protein	Membrane proteins/transport/motility
		YO12_15	Hypothetical protein Fjoh_3665	Unknown
	Down-regulated	YO12_4	Uridylate kinase	Metabolic processes
		YO12_5	MutT/nudix family protein	Transcription/translation
		YO12_10	Translation elongation factor G	Transcription/translation
	Uniquely expressed	YO12_2	Hypothetical protein FB2170_14393	Unknown
		YO12_3	Transaldolase C	Metabolic processes
		YO12_12	Hypothetical protein Plut_1814	Unknown
Biofilm	Up-regulated	YO12_4	Uridylate kinase	Metabolic processes
		YO12_5	MutT/nudix family protein	Transcription/translation
	Down-regulated	YO12_1	OmpA/MotB domain protein	Membrane proteins/transport/motility
		YO12_10	Translation elongation factor G	Transcription/translation
	Uniquely expressed	YO12_6	Inorganic diphosphatase	Metabolic processes
		YO12_7	Alkyl hydroperoxide reductase	Adaptation/protection
		YO12_14	Putative thiol, disulfide interchange protein	Transcription/translation
		YO12_16	Nitrogenase iron protein/ABC transporter	Membrane proteins/transport/motility
Agar surface-associated	Up-regulated	YO12_11	Not identifiable	-
	Down-regulated	YO12_1	OmpA/MotB domain protein	Membrane proteins/transport/motility
		YO12_4	Uridylate kinase	Metabolic processes
		YO12_5	MutT/nudix family protein	Transcription/translation
	Uniquely expressed	YO12_8	Catalase (Peroxidase I)	Metabolic processes
		YO12_9	Spo0B-related GTP binding protein	Transcription/translation
		YO12_13	Glycerol kinase	Metabolic processes

membrane proteins/transport/motility (YO12_16) (Table 3.5). Those unique to the agar surface-associated phase included a putative Spo0B-related GTP binding protein (YO12_9) involved in transcription/translation, and proteins categorized as being associated with metabolic processes (YO12_8 and YO12_13) (Table 3.5).

Protein spots that displayed differential expression included YO12_1, YO12_4, YO12_5, YO12_10, YO12_11, and YO12_15 (Table 3.5). Protein spot YO12_1, the putative OmpA/MotB domain protein, displayed decreased expression in both the biofilm and agar surface-associated phases, while it appeared to be up-regulated in the planktonic phase. Protein spots YO12_4 and YO12_5 displayed increased expression in the biofilm phase, compared to the planktonic and agar surface-associated phases. Protein spot YO12_11, which could not be identified, displayed increased expression in the agar surface-associated phase, compared to the planktonic and biofilm phases. The differentially expressed proteins fell into three general categories, viz., membrane proteins/transport/motility, transcription/translation and metabolic processes (Table 3.5). In the biofilm phase, up-regulated proteins were categorized as being associated with metabolic processes and transcription/translation, while those up-regulated in the planktonic phase were categorized as being involved in metabolic processes or unknown function.

Similarly, 26 proteins were selected from isolate YO64 (Figs. 3.5 and 3.7, Table 3.6) and characterized accordingly (Table 3.7). Seventy-five percent of isolate YO64 protein spectra which could be assigned to a functional category gave positive hits with significant Mowse score values with *F. johnsoniae* proteins previously submitted to protein databases (Table 3.6). While ten protein spots could not be identified, the remaining 16 proteins were assigned to four functional categories (Table 3.7). Protein spots unique to the planktonic phase included a putative heat shock protein Hsp90 (YO64_2) involved in adaptation/protection and proteins categorized as being associated with metabolic processes (YO64_8), transcription/translation (YO64_19 and YO64_21) and proteins unidentifiable (YO64_7 and YO64_23) (Table 3.7). In the agar surface -

TABLE 3.6. Identification of WCP and OMP protein spots/bands of planktonic, biofilm and agar surface-associated phases of *F. johnsoniae*-like isolate YO64

Protein no.	Growth phase	Identified Protein (Gene name)*	Mass (kDa)	pI	Organism	Sequence Coverage (%)	Mowse Score	Significance (Mowse score)	Accession no.	No. of Matched Peptides
YO64_1	Planktonic (WCP)	Heat shock protein Hsp70 (*dnaK*; Fjoh_2631)	67.35	4.78	*Flavobacterium johnsoniae*	25%	123	>74	Q1XU86	2
YO64_2	Planktonic (WCP)	Heat shock protein Hsp90:ATP-binding region, ATPase-like (*hsp*; Fjoh_0260)	70.92	4.96	*Flavobacterium johnsoniae*	25%	85	>74	Q1XR24	2
YO64_3	Planktonic (WCP)	Peptidylprolyl isomerase (*ppiB*; Fjoh_2191)	33.45	5.81	*Flavobacterium johnsoniae*	18%	78	>74	Q1XSZ8	2
YO64_4	Planktonic (WCP)	Superoxide dismutase (*sodB*; Fjoh_1129)	22.43	5.56	*Flavobacterium johnsoniae*	30%	78	>74	Q1XUR5	8
YO64_5	Planktonic (WCP)	Electron transfer flavoprotein beta-subunit (*eftB*; Fjoh_1474)	26.51	5.19	*Flavobacterium johnsoniae*	45%	96	>74	Q1XVQ9	4
YO64_6	Planktonic (WCP)	Succinyl-CoA ligase, alpha subunit (*sucD*; Fjoh_2898)	29.72	5.33	*Flavobacterium johnsoniae*	53%	118	>74	Q1XJ05	2
YO64_7	Planktonic (WCP)	Not Identifiable								
YO64_8	Planktonic (WCP)	Phosphoribosylaminoimidazole-succinocarboxamide synthase (*purC*; Fjoh_2728)	36.09	4.97	*Flavobacterium johnsoniae*	26%	90	>74	Q1XIV1	2
YO64_19	Planktonic (OMP)	RNA binding S1 (*rpsA*; Fjoh_2762)	66.03	5.07	*Flavobacterium johnsoniae*	23%	113	>74	Q1XJ82	2
YO64_20	Planktonic (OMP)	Hypothetical protein Rru_A3216 (Rru_A3216)	116.19	5.32	*Rhodospirillum rubrum*	24%	98	>79	Gi/83594546	3
		PEP-CTERM system TPR-repeat lipoprotein (*pilF*)	95.77	9.22	*Geobacter* spp.	29%	81	>79	gi|191161066	
YO64_21	Planktonic (OMP)	Translation elongation factor Tu: small GTP-binding protein (*tufA*; Fjoh_1936)	43.02	5.26	*Flavobacterium johnsoniae*	24%	92	>74	Q1XPA3	2
YO64_23	Planktonic (OMP)	Not Identifiable								
YO64_9	Biofilm (WCP)	Not Identifiable								
YO64_10	Biofilm (WCP)	Outer membrane chaperone Skp (OmpH) precursor (*skp*; Fjoh_1688)	18.60	8.6	*Flavobacterium johnsoniae*	35%	76	>74	Q1XNK7	2
YO64_11	Biofilm (WCP)	Integrase (*int*)	46.05	9.34	*Bacteroides thetaiotaomicron*	20%	75	>74	Q8A319	3
YO64_12	Biofilm (WCP)	ATP synthase F1, beta subunit (*atpD*; Fjoh_0819)	54.23	4.96	*Flavobacterium johnsoniae*	33%	86	>74	Q1XMW5	2
YO64_13	Biofilm (WCP)	Not Identifiable								
YO64_14	Biofilm (WCP)	Not Identifiable								
YO64_22	Biofilm (OMP)	Translation elongation factor Tu: small GTP-binding protein (*tufA*; Fjoh_1936)	43.02	5.26	*Flavobacterium johnsoniae*	24%	122	>74	Q1XPA4	25
YO64_15	Agar (WCP)	Malate dehydrogenase, NAD-dependent (*mdh*; Fjoh_2255)	32.54	5.54	*Flavobacterium johnsoniae*	20%	75	>74	Q25QU7	4
YO64_16	Agar (WCP)	Cell division protein FtsH ATPase (*ftsH*)	6.54	9.89	*Streptococcus agalactiae*	70%	75	>74	Q3D531	2
YO64_17	Agar (WCP)	Not Identifiable								
YO64_18	Agar (WCP)	Not Identifiable								
YO64_24	Agar (OMP)	Not Identifiable								
YO64_25	Agar (OMP)	Not Identifiable								
YO64_26	Agar (OMP)	Not Identifiable								

* Gene name corresponding to identified protein indicated within ().

TABLE 3.7. Differential expression of identified WCP spots and OMP bands in the planktonic, biofilm and agar surface-associated phases of *F. johnsoniae*-like isolate YO64

Growth Phase	Protein Expression	Protein Spot	Protein ID	Functional Annotation
Planktonic	Up-regulated	YO64_1	Heat shock protein Hsp70	Adaptation/protection
		YO64_3	Peptidylprolyl isomerase	Transcription/translation
		YO64_4	Superoxide dismutase	Adaptation/protection
		YO64_5	Electron transfer flavoprotein beta-subunit	Metabolic processes
		YO64_6	Succinyl-CoA ligase, alpha subunit	Metabolic processes
		YO64_20	PEP-CTERM system TPR-repeat lipoprotein	Membrane proteins/transport/motility
		YO64_21	Translation elongation factor Tu: small GTP-binding protein	Transcription/translation
	Down-regulated	YO64_10	Outer membrane chaperone Skp (OmpH) precursor	Membrane proteins/transport/motility
		YO64_11	Integrase	Adaptation/protection
		YO64_12	ATP synthase F1, beta subunit	Metabolic processes
		YO64_13	Not identifiable	
		YO64_15	Malate dehydrogenase, NAD-dependent	Metabolic processes
		YO64_17	Not identifiable	
	Uniquely expressed	YO64_2	Heat shock protein Hsp90:ATP-binding region	Adaptation/protection
		YO64_7	Not identifiable	
		YO64_8	Phosphoribosylaminoimidazole-succinocarboxamide synthase	Metabolic processes
		YO64_19	RNA binding S1	Transcription/translation
		YO64_23	Not identifiable	
Biofilm	Up-regulated	YO64_10	Outer membrane chaperone Skp (OmpH) precursor	Membrane proteins/transport/motility
		YO64_11	Integrase	Adaptation/protection
		YO64_12	ATP synthase F1, beta subunit	Metabolic processes
		YO64_13	Not identifiable	
	Down-regulated	YO64_1	Glutamate 5-kinase	Adaptation/protection
		YO64_3	Peptidylprolyl isomerase	Transcription/translation
		YO64_4	Superoxide dismutase	Adaptation/protection
		YO64_5	Electron transfer flavoprotein beta-subunit	Adaptation/protection
		YO64_6	Succinyl-CoA ligase, alpha subunit	Metabolic processes
		YO64_17	Not identifiable	
		YO64_22	Translation elongation factor Tu: small GTP-binding protein	Transcription/translation
	Uniquely expressed	YO64_9	Not identifiable	
		YO64_14	Not identifiable	

TABLE 3.7. Continued

Growth Phase	Protein Expression	Protein Spot	Protein ID	Functional Annotation
Agar surface-associated	Up-regulated	YO64_4	Superoxide dismutase	Adaptation/protection
		YO64_15	Malate dehydrogenase, NAD-dependent	Metabolic processes
		YO64_17	Not identifiable	
	Down-regulated	YO64_1	Glutamate 5-kinase	Adaptation/protection
		YO64_3	Peptidylprolyl isomerase	Transcription/translation
		YO64_5	Electron transfer flavoprotein beta-subunit	Adaptation/protection
		YO64_6	Succinyl-CoA ligase, alpha subunit	Metabolic processes
		YO64_10	Outer membrane chaperone Skp (OmpH) precursor	Membrane proteins/transport/motility
		YO64_13	Not identifiable	
	Uniquely expressed	YO64_16	Cell division protein FtsH ATPase	Adaptation/protection
		YO64_18	Not identifiable	
		YO64_24	Not identifiable	
		YO64_25	Not identifiable	
		YO64_26	Not identifiable	

associated phase, a putative cell division protein FtsH (YO64_16) involved in adaptation/protection was uniquely expressed in addition to proteins unidentifiable (YO64_18, YO64_24 - YO64_26) (Table 3.7). Protein spots YO64_9 and YO64_14 uniquely expressed in the biofilm phase were unidentifiable.

Protein spots that displayed differential expression included YO64_1, YO64_3 - YO64_6, YO64_9, YO64_10 - YO64_13, YO64_15, YO64_17, and YO64_20 (Table 3.7). Protein spots YO64_1, YO64_3 YO64_5 and YO64_6, which were up-regulated in the planktonic phase, displayed decreased expression in the biofilm and agar surface-associated phases. Protein spot YO64_4, a putative superoxide dismutase, and the unidentifiable spot YO64_17, displayed increased expression in the agar surface-associated and planktonic phases, compared to the biofilm phase. Protein spots YO64_9 and YO64_11 displayed increased expression in the biofilm phase, compared to the planktonic and agar surface-associated phases. Protein spot YO64_10, a putative outer membrane chaperone Skp, displayed decreased expression in the planktonic and agar surface-associated phases, compared to the biofilm phase. Differentially expressed proteins fell into four general categories, viz., membrane proteins/transport/motility, metabolic processes, transcription/translation and adaptation/protection (Table 3.7). In the planktonic phase, up-regulated proteins were categorized as being involved in adaptation/protection, transcription/ translation, metabolic processes and membrane proteins/transport/motility. In the biofilm phase, up-regulated proteins were categorized as being associated with metabolic processes, adaptation/protection and membrane proteins/ transport/motility, while those up-regulated in the agar surface-associated phase were similarly categorized, except membrane proteins/transport/motility.

3.1. DISCUSSION

Differential protein expression between planktonic and sessile cells is well documented (Kalmokoff *et al*., 2006; Otto *et al*., 2001; Sauer and Camper, 2001;

Sauer *et al*., 2002; Seyer *et al*., 2005; Vilain *et al*., 2004; Vilain and Brözel, 2006). However, there is much debate about biofilm-specific phenotypes and whether the biofilm phenotype can be defined by a specific trend in the proteome of biofilm bacterial cells (Ghigo, 2003; Sauer, 2003; Vilain *et al*., 2004).

In the present study, differences in protein expression between planktonic, biofilm and agar surface-associated growth of *F. johnsoniae*-like isolates were investigated and proteins expressed differentially between the three growth phases and/or uniquely-expressed in each of the three phases were identified. To our knowledge, this is the first study to report on the differences in gene products expressed in planktonic and sessile forms of *F. johnsoniae*-like cells, respectively.

One-dimensional SDS-PAGE WCP and OMP protein profiles obtained for the *F. johnsoniae*-like isolates grown in each of the three phases indicated biofilm and agar surface-associated specific phenotypes, respectively. In the biofilm phase, majority of the isolates demonstrated up-regulated protein expression, in addition to an increase in the number of proteins expressed (Tables 3.2). Variation with respect to protein molecular masses was also observed between planktonic, biofilm and agar surface-associated OMPs of respective isolates following visual analysis (Tables 3.3). However, up-regulation of protein expression in the OMP fraction appeared to be growth phase-dependent for specific isolates and could not be linked to one specific growth phase.

A number of studies have used the high resolving power of 2-D gel electrophoresis to investigate changes in protein expression between the free living and sessile lifestyle of bacteria (Otto *et al*., 2001; Sauer and Camper, 2001; Sauer *et al*., 2002; Seyer *et al*., 2005; Vilain and Brözel, 2006). In the present study this technique has been used to successfully distinguish and investigate the differences in WCP expression between *F. johnsoniae*-like isolates grown planktonicly, as biofilms and during agar surface-associated growth. The intrinsic hydrophobic nature of OMPs make them difficult to solubilize, which often leads to precipitation at their pI values during the isoelectric focusing process (Seyer *et al*., 2005). This reduces the ability of OMPs to enter the second dimension of

separation and valuable information is lost in 2-D gel analysis (Seyer *et al*., 2005). Attempts to optimize 2-D conditions for the OMPs investigated in the present study failed to produce clear 2-D maps and specific OMPs were selected from 1-D gels to investigate OMP differential expression.

The entire proteomes of *F. johnsoniae*-like isolates YO12 and YO64 are not represented in the 2-D WCP electrophoregrams of each growth phase, since low abundance proteins in the pI and Mr range studied and proteins falling outside the selected pI and Mr parameters will not be detected. Nevertheless, differential protein expression was observed in the limited pI pH 4-7 range (Figs. 3.4 - 3.5). Although protein profiles of isolates YO12 and YO64 displayed some percentage of common proteins, significant variation between their proteomes was observed. This might be explained by the genetic and phenotypic diversity displayed by these isolates (Table 3.1).

WCP spots identified in the 2-D maps of isolates YO12 and YO64 displayed homology to several proteins from other bacterial species (Tables 3.4 and 3.6), but the majority of the homologous proteins were either from other *Flavobacterium* spp. or the *F. johnsoniae* UW101 type strain. Qualitative analysis of isolates YO12 and YO64 2-D WCP maps revealed distinct profiles for each respective growth phase and confirmed biofilm and agar surface-associated specific phenotypes in each of these isolates. Due to the differences in protein expression between planktonic, biofilm and agar surface-associated growth, the generated 2-D maps were unsuitable for quantitative analysis using PDQuest software.

Proteins differentially expressed between the planktonic, biofilm and agar surface-associated phases in the present study were classified into the functional categories of metabolic processes, transcription/translation, adaptation/protection and membrane proteins/transport/motility. These categories are similar to what has been observed for differentially expressed proteins in biofilm investigations of other bacteria (Helloin *et al*., 2003; Jouenne *et al*., 2004; Karatan and Watnick, 2009; Prigent-Combaret *et al*., 1999; Sauer and Camper, 2001; Sauer *et al*., 2002; Seyer *et al*., 2005).

When proteins up-regulated or uniquely-expressed in the planktonic phases of isolates YO12 and YO64 were compared, substantial differences were observed in the proteins identified. For isolate YO12, 60% (3/5) appeared to be hypothetical proteins of unknown function, while the remaining two were involved in membrane/transport/motility and metabolic processes, respectively.

Protein YO12_1 displayed significant homology to an OmpA/MotB domain protein (Tables 3.4 and 3.5), an OMP with diverse functions in enterobacterial species (Smith *et al*., 2007). The up-regulation of this protein in YO12 planktonic cells is in accordance with the findings of Otto *et al*. (2001), where OmpA, one of the major OMPs of *E. coli*, was shown to be down-regulated in abiotic surface-associated cells and up-regulated in planktonic cells of *E. coli*. In *E. coli*, OmpA supports the structural integrity of the outer membrane, contributing to cell shape, and it is also involved in cell-cell interactions and host invasion of this organism (Otto *et al*., 2001; Smith *et al*., 2007). Unlike other major OMPs, such as OmpR in *E. coli* and OprF in *P. aeruginosa* (Otto *et al*., 2001; Seyer *et al*., 2005), OmpA does not seem to play a role in biofilm formation, but is implicated in invasion by pathogenic *E. coli* strains (Otto *et al*., 2001; Smith *et al*., 2007). Down-regulation of this protein in biofilm cells does not seem to be restricted to *E. coli*, since De Vriendt *et al*. (2005) also observed significant down-regulation of OmpA in *Shewanella oneidensis* biofilm cells when compared to planktonic cells. In *F. psychrophilum*, OmpA constitutes a major outer membrane glycoprotein capable of inducing antibody responses in fish (Dumetz *et al*., 2007). Glycoproteins have also been shown to mediate adherence of virulent *F. psychrophilum* (Møller *et al*., 2003) and *F. columnare* (Decostere *et al*., 1999a) strains to fish tissue as part of the invasion process. Although this protein seemed down-regulated in the agar surface-associated phase (Table 3.5), it was still expressed at higher levels compared to biofilm cells (Fig. 3.4 C). Up-regulation and/or expression of this protein in the planktonic and agar surface-associated phases may be directly related to host-invasion and virulence of *F. johnsoniae*-like isolate YO12, rather than attachment to abiotic surfaces.

Protein YO12_3 displayed significant homology to transaldolase C (Tables 3.4 and 3.5), an enzyme of the non-oxidative pentose phosphate cycle. Bacterial transaldolases are responsible for the catabolism of pentose sugars (Sprenger *et al*., 1995). The abundance of a putative transaldolase in the planktonic phase of this isolate may be an indication of carbon flux through the pentose phosphate pathway (Sprenger *et al*., 1995). The unique expression of a putative transaldolase may result in metabolism of pentose sugars which form part of ECC and capsular material in agar surface-associated cells, leading to the decreased concentrations of ECC (480 nm) observed in broth cultures (planktonic cells) of isolate YO12 (Chapter 2; Table 2.4).

By contrast, up-regulated or uniquely-expressed proteins of planktonic YO64 cells were predominantly adaptation/protection (25%), transcription/translation (25%), and metabolic processes (25%), while 8% (1/12) was associated with membrane/transport/motility and 17% (2/12) were unidentifiable. Up-regulated proteins involved in adaptation/protection included a putative heat shock protein and SOD, while a putative heat shock protein was uniquely expressed (Table 3.7). Protein YO64_1 and protein YO64_2 displayed significant homology to the heat shock proteins, Hsp70 and Hsp90, respectively, which are cellular chaperones that participate in protein refolding. Heat shock proteins may be induced by nutrient limitation, cold shock or oxidative stress and form part of the general stress response in many microorganisms (Boorstein and Craig, 1990; Lelivelt and Kawula, 1995; Nyström, 2004). Up-regulation of Hsp70 was observed in stationary phase *E. coli* cells (Nyström, 2004) and also in nutritionally deprived *Saccharomyces cerevisiae* cells (Boorstein and Craig, 1990). Heat shock proteins also prevent the aggregation of damaged protein complexes and under conditions of stress, expression of Hsp90 (uniquely expressed in YO64 planktonic cells) has been shown to increase up to 10-fold (Buchner, 1999). Over-expression of these proteins in stationary phase planktonic cells of isolate YO64 may be responsible for the up-regulation of the putative Hsp70 and/or unique presence of the putative Hsp90. The differential

and unique expression of putative stress response proteins is likely to be part of the stationary phase response.

Protein YO64_4 displayed significant homology to a SOD, an enzyme that converts O_2^- to H_2O_2, which is transformed to H_2O by catalase, thereby playing a role in detoxification (Helloin *et al*., 2003). It is induced in periods of oxidative stress and shown to be up-regulated during carbon starvation, such as glucose starvation, in planktonic cells (Helloin *et al*., 2003) and in growth-arrested cells (Dukan and Nyström, 1998).

The up-regulated proteins involved in transcription/translation included putative molecular chaperones, peptidylprolyl isomerase and EF-Tu, while a putative RNA-binding protein was uniquely expressed (Table 3.7). Protein YO64_3 displayed significant homology to peptidylprolyl isomerase, a general stress response protein, functioning in the repair and refolding of damaged proteins (Helloin *et al*., 2003). Contrary to what was observed in this study, De Vriendt *et al*. (2005) found a peptidylprolyl isomerase that was significantly up-regulated in *S. oneidensis* biofilm cells, compared to planktonic cells. Protein YO64_21 displayed significant homology to the translation elongation factor Tu (small GTP-binding protein). This over-abundant cellular protein, partly associated with the inner membrane, is involved in peptide chain formation and with chaperone-like functions where it forms complexes with unfolded proteins, increasing the refolding of unfolded proteins and protecting proteins against thermal denaturation (Caldas *et al*., 1998). EF-Tu may exert multiple roles during conditions of stress; it may even abort the production of redundant proteins and induce translation of valuable stress proteins (Helloin *et al*., 2003). In *E. coli*, EF-Tu comprises 5-10% of the cellular proteome (Caldas *et al*., 1998) and may regulate the transcription and translation of proteins during periods of nutritional deprivation (Helloin *et al*., 2003). This protein is membrane-bound in *E. coli* during glucose, galactose, glutamate, nitrogen and/or phosphate deprivation (Young and Bernlohr, 1991), which explains the presence of this protein in the OMP fraction of stationary phase YO64 cells.

Protein YO64_19 displayed significant homology to the RNA-binding protein S1, a protein associated with the ribosome where it binds to RNA and initiates the translation of several mRNAs. The putative function of cold shock proteins involved in RNA metabolism has been suggested to be similar to that of the RNA binding protein S1 (Graumann and Marahiel, 1998). The putative RNA binding protein S1 was possible cytoplasmic contamination, since it is usually found in association with the 30S ribosomal subunit (Sorensen *et al*., 1998). It is not unusual to obtain contamination of cytoplasmic proteins in the outer membrane fraction and Seyer *et al*. (2005) reported that 9% of identified proteins from their OMP purification comprised of cytoplasmic proteins.

Proteins involved in metabolic processes included two up-regulated proteins, a putative electron transfer flavoprotein beta-subunit and succinyl-CoA ligase, while a putative phosphorribosyl-aminoimidazole-succinocarboxamide (SAICAR) synthase, was uniquely expressed. Protein YO64_5 displayed significant homology to an electron transfer flavoprotein beta-subunit. It is likely to be involved in intermediary metabolism, since electron transfer proteins facilitate the overall oxidation-reduction balance in cells (Scott and Ludwig, 2004). Protein YO64_6 displayed significant homology to succinyl-CoA ligase, an enzyme involved in the tricarboxylic acid (TCA) cycle. The abundance of this protein in stationary phase cells suggests a carbon flux in the TCA cycle, which may indicate a fraction of actively dividing cells within the stationary phase population.

Protein YO64_8 displayed significant homology to SAICAR synthase, an enzyme involved in purine nucleotide biosynthetic pathways. SAICAR synthase catalyzes the conversion of 5'-phosphoribosyl-5-aminoimidazole-4-carboxylic acid and aspartic acid to SAICAR (Beyhan *et al*., 2006).

Protein YO64_20, involved in membrane/transport/motility, displayed significant homology to a hypothetical protein and a PEP-CTERM system TPR-repeat lipoprotein. The TPR-repeat lipoprotein belongs to the PEP-CTERM protein family, a family of proteins associated with Gram-negative soil bacteria capable of outer membrane and EPS production (Haft *et al*., 2006; Norton *et al*.,

2008). A number of PEP-CTERM proteins have been detected in *Rhodospirillum rubrum*, and it is likely that the identified hypothetical protein Rru_A3216 belongs to this family, since several Pro-X-Pro motifs and a Pro-Glu-Glu-Pro motif, close to the near-invariant Pro-Glu-Pro motif of the PEP-CTERM domain, were present in the C-terminal of this protein sequence (Haft *et al*., 2006). These proteins facilitate the transport of molecules, including proteins, across the cell membrane and are generally associated with bacteria, especially proteobacteria, from aquatic sediments and soils, and biofilms (Haft *et al*., 2006; Norton *et al*., 2008). The TPR structural motif is also associated with a wide range of proteins that assemble membrane-bound multi-protein complexes (D'Andrea and Regan, 2003). In *P. aeruginosa* the outer membrane lipoprotein PilF, a protein essential for type IV pili biogenesis was found to have 6 consecutive TPR-like repeats (Koo *et al*., 2008). Besides playing a role in virulence and motility of *P. aeruginosa*, type IV pili also play an important role in cell attachment and biofilm formation of this organism (Koo *et al*., 2008; Van Schaik *et al*., 2005). In *Myxococcus xanthus*, proteins with TPR-repeats have been associated with A motility, a type of gliding motility that depends on the polar excretion of slime (Yu and Kaiser, 2007). Lipoproteins have been found to play a key role in gliding motility of *F. johnsoniae* and disruption of five Gld lipoproteins localized in the cytoplasmic and outer membrane resulted in loss of motility (McBride, 2004). However, the function of these proteins in biofilm formation has not been elucidated. Interestingly, proteins of the PEP-CTERM protein family have not been described previously in members of the genus *Flavobacterium* or in any animal pathogens yet (Haft *et al*., 2006). This is the first description, to our knowledge, of a putative PEP-CTERM protein in *F. johnsoniae* and specifically *F. johnsoniae*-like isolate YO64, responsible for fish disease and capable of biofilm formation (Bernardet and Bowman, 2006; Flemming *et al*., 2007). The function of this protein in flavobacterial biofilm formation is unknown and requires further investigation.

During carbon starvation of bacterial cells, as occurs in the stationary phase, proteins involved in transport and metabolism of nucleic acids and

available carbon sources are up-regulated as a means of survival (Helloin *et al*., 2003). Several putative proteins with metabolic functions were up-regulated and/or uniquely expressed in the planktonic stationary phase of both *F. johnsoniae*-like isolates YO12 and YO64. Putative stress-response proteins and chaperones very similar to those described by Blokpoel *et al*. (2005) for *Mycobacterium smegmatis* stationary phase cells were also observed in the stationary phase planktonic cells of isolate YO64. The diversity in putative proteins involved in metabolism, stress-response and transcriptional factors, such as the putative EF-TU, indicated a physiologically heterogeneous mixture of planktonic cells in the stationary phase of isolate YO64, consisting of actively dividing, transient and growth-arrested subpopulations (Blokpoel *et al*., 2005; Helloin *et al*., 2003).

The biofilm mode of growth seems to represent the preferred growth form of most aquatic bacteria (Davies, 2000). However, biofilm formation is often described as an adaptive response to environmentally challenging conditions (Bell, 2001; Donlan, 2002; Jefferson, 2004; Stickler, 1999; Webb *et al*., 2003). Accordingly, Jouenne *et al*. (2004) proposed three main classes of proteins commonly differentially expressed during biofilm formation, viz., membrane proteins, proteins involved in metabolic processes and stress-response proteins associated with adaptation and protection.

Although up-regulated or uniquely expressed proteins with similar functional properties were observed in the biofilm phases of isolates YO12 and YO64, the identified proteins were distinct for each isolate. For isolate YO12, 43% were functional as putative transcriptional regulators, while others were involved in metabolic processes, adaptation/protection and membrane/transport/ motility. Up-regulated proteins involved in transcription/translation included a putative MutT/nudix family protein and translation elongation factor G, while a putative thiol, disulfide interchange protein was uniquely expressed (Table 3.5). Protein YO12_5 displayed significant homology to a conserved hypothetical protein with homology to a MutT/nudix family protein. The MutT protein in *E. coli* has anti-mutational functions, regulating oxidized guanine as part of the substrate

dGTP and chromosomal DNA (Fowler and Schaaper, 1997). MutT is a nucleoside triphosphatase hydrolyzing dGTP and 8-oxodGTP to dGMP and pyrophosphate, thus regulating cellular dGMP levels (Fowler and Schaaper, 1997). In many bacteria, GTP and GMP levels are regulated as part of the ribonucleic acid second messenger c-di-GMP regulatory system, which influences biofilm formation, swarming motility and virulence, amongst other cellular functions (Beyhan *et al*., 2007; Simm *et al*., 2004). However, due to the absence of genes encoding c-di-GMP regulatory proteins, diguanylate cyclases and phosphodiesterases, in the genome sequence of *F. johnsoniae* UW101, the c-di-GMP signaling system is unlikely to be used by this organism (McBride *et al*., 2009).

Protein YO12_10 displayed significant homology to the translation elongation factor G, a transcriptional factor participating in the elongation process of protein biosynthesis (Martemyanov and Gudkov, 2000). Similar to eEF2 in eukaryotes, EF-G catalyzes the translocation process of tRNAs when the ribosome switches from the pre-translocational to the post-translocational state in prokaryotic organisms (Spahn *et al*., 2004). However, EF-G may also function as a chaperone during conditions of stress (Kalmokoff *et al*., 2006). EF-G was also demonstrated to be significantly up-regulated in *Campylobacter jejuni* biofilm cells (Kalmokoff *et al*., 2006). Up-regulation of transcriptional factors, such as EF-G, in biofilm cells most likely indicates that biofilm growth consists of a large group of actively dividing cells (Kalmokoff *et al*., 2006).

Protein YO12_14 displayed significant homology to a putative thiol, disulfide interchange protein. In Gram-negative bacteria, protein disulfide isomerases (thiol:disulfide oxidoreductases) function as protein folding catalysts and are required for thiol-disulfide exchanges, the reactions taking place during disulfide bond formation and rearrangement in proteins (Missiakas and Raina, 1997). Thiol:disulfide oxidoreductases share a similar active site, the conserved Cys-X-X-Cys motif (Fabianek *et al*., 2000; Missiakas and Raina, 1997; Nakamoto and Bardwell, 2004), which is also present (C-G-P-C) in the putative thiol:disulfide interchange protein sequence to which protein YO12_14 showed

significant homology. Exported proteins contain disulfide bonds contributing to the stability of their tertiary structure and, in some, catalytic activity (Missiakas and Raina, 1997). Thiol:disulfide oxidoreductases can be found in the periplasm or they may be membrane-bound in bacteria where they play an integral role in the folding pathway of many exported proteins, including proteins required for virulence and bacterial adhesion (Álvarez *et al*., 2006; Missiakas and Raina, 1997; Peek and Taylor, 1992; Stenson and Weiss, 2002; Yu and Kroll, 1999). Therefore, it is not surprising to find this protein in the OMP fraction of isolate YO12. In *F. psychrophilum*, disruption of the *tlpB* gene (first locus in the *fpt* operon), encoding a putative thiol:disulfide oxidoreductase, was shown to inhibit gliding motility, extracellular proteolytic activity, cytotoxicity and virulence, but increased the biofilm-forming ability in this organism (Álvarez *et al*., 2006). The *tpiA* gene, the third locus of the *fpt* operon, was uninterrupted and encodes a triosephosphate isomerase which was shown to be specifically expressed during *Staphylococcus aureus* biofilm formation (Álvarez *et al*., 2006). In a study performed by Mangalapalli-Illathu *et al*. (2008), the *S. enterica* biofilm phenotype displayed a more efficient adaptive response to benzalkonium chloride compared to planktonic cells, and a thiol:disulfide interchange protein was up-regulated in adapted biofilm cells, whereas it remained unchanged in adapted planktonic cells. Yu and Kroll (1999) showed that DsbA, a thiol:disulfide oxidoreductase, played an important role in the biogenesis of pili, such as P pili and type IV pili, and other adhesins necessary for bacterial attachment and surface colonization and the production of toxins and components of the type III secretion system, a multi-component protein complex spanning the membrane for the excretion of virulence factors. Interestingly, *E. coli* DsbA mutant cells displayed impaired biofilm formation and reduced attachment to both abiotic and biotic surfaces, compared to wild-type cells (Lee *et al*. 2008). This indicates some relationship between thiol:disulfide oxidoreductases and related proteins and cellular attachment and/or biofilm development. *F. johnsoniae* does not produce pili, however, thin filaments largely associated with gliding motility, have been detected on the cell surface of this organism (Liu *et al*., 2007). Thiol:disulfide

oxidoreductases may play a role in the folding and structure configuration of these cell-surface filaments, however, the role of these appendages in bacterial attachment and other behavioural processes, such as virulence, has not been investigated yet. Therefore, protein YO12_14, a putative thiol:disulfide oxidoreductase, may play an important role in motility, virulence and fish host invasion of *F. johnsoniae*-like isolates, but the relationship of this protein with biofilm formation is still poorly understood.

Proteins involved in metabolic processes included an up-regulated, putative uridylate kinase, while a putative inorganic diphosphatase was uniquely expressed (Table 3.5). Protein YO12_4 displayed significant homology to an uridylate kinase, an enzyme required for the conversion of UMP to uridine diphosphate (UDP), the substrate precursors of uracil and uridine-5'-triphosphate (UTP), respectively. Uracil and UTP function as ribonucleic acid signal molecules, important biological regulators in eukaryotic and prokaryotic organisms (Baker and Kelly, 2004; Lazarowski and Boucher, 2001; Ueda *et al*., 2009). For instance, UTP functions as an extracellular signal molecule in humans (Lazarowski and Boucher, 2001) and uracil forms part of an important cell-signaling system in *P. aeruginosa* (Ueda *et al*., 2009). More specifically, UTP regulates cellular functions through a membrane receptor, which in turn regulates phospholipases and mitogen-activated protein kinases (Lazarowski and Boucher, 2001), whereas uracil influences all three known quorum sensing pathways in *P. aeruginosa* and regulates biofilm formation, swarming and virulence (Ueda *et al*., 2009). Ueda *et al*. (2009) showed that uracil, uridine monophosphate (UMP) and uracil phosphoribosyltransferase (Upp) are all required for quorum sensing phenotypes in *P. aeruginosa*. The enzyme Upp and substrates, UTP, UMP and uracil, are all part of the uracil synthesis pathway. WCP YO12_4 expression in the biofilm phase of isolate YO12 appeared to be up-regulated. This may indicate possible uracil, UMP or UTP regulation of quorum sensing, or other cell-signalling pathways required for biofilm formation and/or other behavioural characteristics, such as virulence and motility, in this *F. johnsoniae*-like isolate. Putative proteins likely to play a role in cell

signaling/response regulatory systems may indicate regulated biofilm development in the *F. johnsoniae*-like isolates. The up-regulation of a putative uridylate kinase, regulating dUMP levels in the biofilm phase of *F. johnsoniae*-like isolate YO12, may signify the presence of ribonucleic acid second messenger systems, such as the UMP-related regulatory system (Caiazza *et al*., 2007; Simm *et al*., 2004; Ueda *et al*., 2009), influencing *F. johnsoniae*-like isolate attachment responses.

Protein YO12_6 displayed significant homology to an inorganic diphosphatase, an enzyme with similar functional properties to MutT. Inorganic diphosphatases commonly catalyze the hydrolysis of phosphorus bonds from nucleotides to produce pyrophosphate (diphosphate) and nucleoside monophosphates, such as dGMP and dUMP (Abeijon *et al*., 1989; Li *et al*., 2003).

Protein YO12_7, involved in adaptation/protection, displayed significant homology to an alkyl hydroperoxide reductase (thiol-specific antioxidant) (Tables 3.4 and 3.5), an enzyme that plays a role in the removal of reactive oxygen species, such as H_2O_2, during the oxidation of thiol compounds by thiol:disulfide oxidoreductases (Missiakas and Raina, 1997). As part of the oxidative stress response, anti-oxidants, including alkyl hydroperoxidase reductases, were significantly up-regulated in biofilm cells of *Candida albicans* (Seneviratne *et al*., 2008) and *C. jejuni* (Kalmokoff *et al*., 2006). Differences in redox potential across the depth of the biofilm may lead to significant up-regulation of alkyl hydroperoxidase reductases, thereby preventing reactive oxygen species to cause damage to the biofilm cells (Seneviratne *et al*., 2008).

Protein YO12_16, involved in membrane/transport/motility, displayed significant homology to a nitrogenase iron protein/ABC transporter (Tables 3.4 and 3.5) and is likely to be a membrane-bound nitrogenase or membrane-associated transporter with nitrogenase activity (Georgiadis *et al*., 1992; Schmehl *et al*., 1993). Nitrogenase genes and proteins are associated with many prokaryotes, including Gram-negative and Gram-positive bacteria, and have been found to be very similar, despite the phylogenetic diversity of bacteria that

fix molecular nitrogen and harbour nitrogenase components (Hennecke *et al*., 1985). The nitrogenase enzyme complex consists of two protein components, viz., the Fe protein or dinitrogenase reductase and the molybdenum-iron protein, MoFe, or dinitrogenase (Hennecke *et al*., 1985). One class of nitrogen fixation proteins constitute membrane-associated transporters involved in electron transport to nitrogenase. These transporter systems generally consist of an inner membrane-localized ATPase, membrane fusion protein and outer membrane protein component (Hinsa *et al*., 2003). The nitrogenase iron protein to which protein YO12_16 showed significant homology was classified as an ATP-binding cassette (ABC) ATPase due to the presence of an ABC transporter nucleotide-binding domain. ABC transporters represent a family of proteins involved in the transport of many compounds, such as ions, sugars, toxins, hydrolytic enzymes, large surface bound proteins and extracellular polysaccharides (Hinsa *et al*., 2003; Holland *et al*., 2005). Transporter systems can be essential components in communication of bacteria with the surrounding environment and they are known to play a role in bacterial adherence and biofilm formation (Hinsa *et al*., 2003; Silver *et al*., 2001). Analysis of OMP profiles in sessile *E. coli* cells revealed uniquely expressed OMPs involved in iron transport systems, such as ABC transporters, for the uptake of iron required for cellular processes, including respiration, RNA synthesis and the removal of reactive oxygen species (Rivas *et al*., 2008). However, the role of these proteins and transporters in biofilm formation of *E. coli* is unknown (Rivas *et al*., 2008). In *Pseudomonas fluorescens*, an ABC transporter was found to play an important role in biofilm formation through the excretion of a large membrane bound protein, LapA, which was required for the transition between reversible and irreversible attachment during early biofilm development (Hinsa *et al*., 2003). Similarly, LapA mutants of *P. putida* also lacked the ability to adhere to abiotic surfaces (Hinsa *et al*., 2003). Moreover, certain members of the ABC transporter superfamily are involved in the export of capsular material and EPS (Silver *et al*., 2001). Interestingly, isolate YO12 showed increased ECC production in the agar surface-attached phase, compared to other *F. johnsoniae*-like isolates (Chapter 2). The *F.*

johnsoniae gliding motility genes *gldA*, *gldF* and *gldG* are thought to form an ABC transporter required for gliding motility (Nelson and McBride, 2006). Loss of the GldF component of the transporter was linked to a loss in membrane-dense areas and cell surface filaments associated with motility, leaving *gldF* mutants immotile (Liu *et al*., 2007). The membrane dense areas may represent an abundance of the ABC transporter components, including GldF, anchoring the cell-surface filaments (Liu *et al*., 2007). Alteration of these cell surface components in *F. johnsoniae* primarily influence gliding motility, however, they may potentially play a role in bacterial attachment and/or influence biofilm formation. The unique presence of the putative nitrogenase iron protein in the biofilm phase suggests a role for the ABC transporter system in bacterial attachment, EPS production and/or biofilm formation of isolate YO12.

For isolate YO64, up-regulated proteins in the biofilm phase were involved in membrane/transport/motility, adaptation/protection and metabolic processes, respectively, while the expression of the putative transcriptional regulator EF-Tu seemed down-regulated. Protein YO64_10, involved in membrane/transport/ motility, displayed significant homology to an OmpH precursor (Tables 3.6 and 3.7). OMPs of bacterial pathogens often play an important role in virulence and generally stimulate an immune response during the infection process (Dumetz *et al*., 2006). An OmpH-like surface antigen was detected in *F. psychrophilum* and was shown to induce a strong immune response in trout (Dumetz *et al*., 2006). Although OmpH has been described as a molecular chaperone involved in the correct folding of other OMPs in the periplasmic space and insertion of these proteins into the outer membrane of bacteria, in *F. psychrophilum* its role involves an interaction function with the surrounding environment (Dumetz *et al*., 2006). The OmpH protein detected in *F. johnsoniae*-like isolate YO64 may be surface-bound and its increased expression in the biofilm phase may indicate a possible relationship between expression of this protein and biofilm formation. In *E. coli*, OmpH plays a key role during the biogenesis of the OMPs, LamB and OmpA, and a mutant cell lacking OmpH displayed an over-all reduction in OMPs (Schäfer *et al*., 1999). OmpH in *F. johnsoniae* and other *Flavobacterium* spp.

may also be involved in the folding and outer membrane insertion of OMPs required for adherence and/or biofilm formation. Therefore, OmpH may be involved in cell adherence directly or indirectly by playing a role in the biogenesis of other OMPs in this *F. johnsoniae*-like isolate. However, the exact function of this protein in *F. johnsoniae* and biofilm formation requires further investigation.

Protein YO64_11, involved in adaptation/protection, displayed significant homology to an integrase (Tables 3.6 and 3.7), which may indicate an increased frequency of horizontal gene transfer in the biofilm mode of this organism. It has been proposed that the biofilm lifestyle, a microbial growth adaptation generally associated with stressful conditions, facilitates lateral and horizontal gene transfer and large amounts of DNA have been detected in EPS within biofilms (Vilain and Brözel, 2006; Whitchurch *et al*., 2002). Integrases have been associated with antibiotic resistance genes and pathogenicity determinants, such as pathogenicity islands (Hacker and Kaper, 2000; Walsh *et al*., 2005). *F. johnsoniae* is known to harbour antibiotic resistance genes, such as β-lactamases (Naas *et al*., 2003), however, although β-lactamases associated with *Bacteroides* spp. are transferable by mobile elements, it is not known whether these genes were/are carried by integrons in *F. johnsoniae* (Walsh *et al*., 2005). Pathogenicity islands have not been described previously in *Flavobacterium* spp., however, pathogenicity determinants, such as metalloproteases and other proteolytic enzymes and surface adhesins, such as glycoproteins, have been shown to play a major role in *F. psychrophilum* and *F. columnare* pathogenicity (Bernardet and Bowman, 2006; Decostere *et al*., 1999a-b; Secades *et al*., 2001; Secades *et al*., 2003). It might be speculated that the increased expression of the putative integrase in the biofilm phase of *F. johnsoniae*-like isolate YO64 may increase the frequency for the exchange and uptake of foreign DNA, facilitating the survival of the organism under stressful conditions, i.e., an adaptive response.

Protein YO64_12, involved in metabolic processes, displayed significant homology to an ATP synthase F1, beta subunit, which forms part of F1-ATPase involved in ATP metabolism and energy production. The F1-ATPase functions

as a rotary motor, hydrolyzing ATP through its beta subunits (Itoh *et al*., 2004). Although Schembri *et al*. (2003) observed an 11-fold change of the ATP synthase F1 gamma-subunit in biofilm cells of *E. coli*, compared to logarithmic and stationary phase planktonic cells, the reason for up-regulation of these F1-ATPase components is unclear.

Protein YO64_22 displayed significant homology to the EF-Tu identified in the planktonic phase (YO64_21). Although EF-Tu was shown to be highly up-regulated in *Streptococcus mutans* biofilm cells (Svensäter *et al*., 2001), this protein seemed down-regulated in the biofilm phase of isolate YO64. The reason for possible down-regulation of this protein in the biofilm phase is unclear. Electrophoretic conditions of the 1-D gels may have resulted in a separated protein band (Fig. 3.7), which could explain the lower expression level observed.

The putative proteins up-regulated and/or uniquely expressed in the biofilm phase of the *F. johnsoniae*-like isolates YO12 and YO64 could be classified into the three major classes described by Jouenne *et al*. (2004), viz., membrane proteins, metabolic processes and adaptation/protection, as well as a fourth class, transcription/translation. Helloin *et al*. (2003) demonstrated that the adaptive response of *E. coli* biofilm cells involved the up-regulation of transcription and translational factors. There seems to be a need to include proteins involved in transcription and translational regulation as an adaptive measure in the biofilm mode of growth (Helloin *et al*., 2003).

The putative proteins belonging to the metabolic processes functional category, which were up-regulated and/or uniquely expressed in biofilm cells of isolates YO12 and YO64, differed from those expressed by their stationary phase planktonic counterparts. Biofilm-specific phenotypic traits indicate that *F. johnsoniae*-like biofilm cells do not represent stationary phase planktonic cells and although factors with similar functional categories were demonstrated in both phases, their biofilm proteomes were distinct, with some aspects of commonality.

Interestingly, membrane-associated proteins with potential biofilm-related functions were up-regulated and/or uniquely expressed in the biofilm phase of *F. johnsoniae*-like isolates YO64 and YO12, respectively. These proteins may well

influence bacterial attachment by facilitating cell adherence or the excretion of EPS required for irreversible attachment and microcolony formation (Donlan, 2002; Hall-Stoodley and Stoodley, 2002; Otto *et al*., 2001; Seyer *et al*., 2005; Stanley and Lazazzera, 2004; Tsuneda *et al*., 2003; Van Houdt and Michiels, 2005; Wang *et al*., 2004b). As demonstrated for *P*. *aeruginosa* (Sauer *et al*., 2002), the changes observed in the biofilm proteome of the *F*. *johnsoniae*-like isolates were complex and may be regulated by cell signaling mechanisms or two component regulatory systems.

There has been speculation as to whether agar surface-associated growth and biofilm growth are phenotypically distinct entities. Donlan (2002) and Mikkelsen *et al*. (2007) have argued that agar surface-associated colonies behave like planktonic cells which are stranded on a solid surface. Mikkelsen *et al*. (2007) showed that protein profiles of agar surface-associated growth of *P*. *aeruginosa* cells resemble those of planktonic growth and that the biofilm proteome is distinct. Kolter and Greenberg (2006) on the other hand, described *P*. *aeruginosa* colonies as being air-exposed biofilms. In other bacterial species, such as the soil bacterium *Bacillus subtilis*, agar surface-associated growth has been used as model system for the study of biofilm formation and it was suggested that colonies that grow on semi-solid media can be considered to represent a form of biofilm (Branda *et al*., 2004; Hamon and Lazazzera, 2001; Morikawa *et al*., 2006). Examination of the YO12 and YO64 agar surface-associated proteome and comparison with planktonic and biofilm proteomes suggested that they are a type of biofilm.

Substantial differences were also observed in the limited number of up-regulated and/or uniquely expressed proteins selected from the agar surface associated phases of isolates YO12 and YO64. For isolate YO12, 67% were involved in metabolic processes, including a putative catalase and glycerol kinase, while the remaining protein was functional as a putative transcriptional regulator.

Protein YO12_8 displayed significant homology to catalase (peroxidase I) (Tables 3.4 and 3.5), an enzyme responsible for detoxification by removing

oxygen radicals, such as the conversion of H_2O_2 to H_2O. During the infection process of pathogenic organisms, oxidative stress is self-evident and pathogenic organisms employ versatile antioxidant defense enzymes, such as peroxidases, to remove oxygen radicals and to maintain a reducing state in the periplasmic or cytoplasmic environment (Shin *et al*., 2008). In *P. aeruginosa*, catalase is important for peroxide-resistance and osmoprotection and it is required for full virulence in this organism (Shin *et al*., 2008). However, enzymes with peroxidase activity have also been implicated in stress response within biofilms (Shin *et al*., 2008). Catalase has been shown to reduce the susceptibility of Gram-negative bacteria to H_2O_2 in the biofilm state (Mai-Prochnow *et al*., 2008). The major H_2O_2-scavenging catalase, KatA, was found to play a critical role in *P. aeruginosa* biofilm homeostasis, and its metastability and extracellular presence seemed to increase *P. aeruginosa* biofilm peroxide-resistance, compared to other catalases (Shin *et al*., 2008). The putative catalase in the agar surface-associated phase of *F. johnsoniae*-like isolate YO12 may also play a role in biofilm-dependent peroxide-resistance and/or virulence in this organism.

Protein YO12_13 displayed significant homology to glycerol kinase (Tables 3.4 and 3.5), an enzyme involved in glycerol metabolism. Glycerol kinase catalyzes the phosphorylation of glycerol to produce glycerol-3-phosphate, an important component of phospholipid biosynthesis (Sakasegava *et al*., 2003). Interestingly, glycerol-3-phosphate was shown to resuscitate dormant, persister cells in *E. coli* biofilms, giving rise to actively dividing cells (Spoering *et al*., 2006). However, phospholipids may also form part of the hydrophilic-rich layer underlining the capsule, as observed in sliding and biofilm-forming *M. smegmatis* cells (Recht and Kolter, 2001). Isolate YO12 displayed a strong gliding ability and increased production of ECC on agar compared to planktonically grown cells (Table 2.4), which may be related to the production of thick capsule in the agar surface-associated phase. Thus, unique expression of a putative glycerol kinase in the agar surface-associated phase may be required for the synthesis of capsular material in *F. johnsoniae*-like isolate YO12.

Protein YO12_9 displayed significant homology to the Spo0B-related GTP binding protein (Tables 3.4 and 3.5), a phosphotransfer protein involved in regulated sporulation systems, such as the Spo0A-related system required for biofilm formation by *B. subtilis* on agar surfaces (Branda *et al*., 2001; Hamon and Lazazzera, 2001). Mutant cells of *B. subtilis* defective in Spo0A (sporulation transcription factor), Spo0F (response regulator receiving phosphate from kinases) and/or Spo0B (phosphotransfer protein transferring phosphate from Spo0F to Spo0A) display defects in biofilm formation, respectively (Hamon and Lazazzera, 2001). Biofilm formation of isolate YO12, although weak on abiotic surfaces, may be influenced by a response regulatory system involving transcriptional factors similar to the sporulation system in *B. subtilis*.

Up-regulated and/or uniquely-expressed proteins of agar surface-associated YO64 cells were predominantly unidentifiable (62%), while 25% (2/8) were involved in adaptation/protection and 12% (1/8) was involved in metabolic processes. Of the proteins involved in adaptation/protection, a putative SOD (YO64_4 described in planktonic phase) was up-regulated, while the putative cell division protein FtsH ATPase was uniquely expressed (Tables 3.6 and 3.7). Protein YO64_16 displayed significant homology to the cell division protein, FtsH ATPase, an AAA metalloprotease, which belongs to the AAA protein superfamily, consisting of ATPases involved in diverse cellular processes, such as protein transport, cell cycle and gene expression control, and proteolytic activity (Zellmeier *et al*., 2003). This metalloprotease is typical of eubacteria, in which it controls the biogenesis of membrane-bound proteins and degrades short-lived cytoplasmic regulatory proteins (Zellmeier *et al*., 2003). *B. subtilis* mutants lacking FtsH display abnormal cell growth, including filamentous forms, mainly associated with a defect in the production of penicillin-binding proteins and other uncomplexed membrane proteins (Zellmeier *et al*., 2003). In the FtsH mutant, filamentous growth of *B. subtilis* was shown to be directly associated with an increased production of the penicillin-binding protein PBP4 (Zellmeier *et al*., 2003). The absence of the FtsH protein in liquid-grown cells of this *F. johnsoniae*-like isolate may explain the longer, filamentous cell morphology

associated with this growth form, as opposed to shorter, pleomorphic cells in the agar surface-associated phase (Flemming, 2006). Penicillin-binding proteins and other proteins involved in peptidoglycan structure and biosynthesis have been shown to play an important role in the cell envelope integrity of *Streptococcus gordonii* biofilm cells (Loo *et al*., 2000).

Since FtsH regulates cell membrane-associated proteins, including those associated with peptidoglycan structure, it could potentially play a role in attachment and/or biofilm formation of *F. johnsoniae*-like isolates. FtsH may also be involved in transcriptome and proteome regulation, thereby participating in regulatory networks that control biofilm formation (Simionato *et al*., 2006). Similar to FtsH, the cell division protein FtsZ was up-regulated and/or highly abundant in biofilm cells of *S. mutans* (Svensäter *et al*., 2001) and *L. monocytogenes* (Hefford *et al*., 2005), respectively.

Another member of the AAA protein superfamily, the Clp ATPase family, has been shown to play a major role in biofilm formation and virulence of *S. aureus* (Frees *et al*., 2004). Similar to FtsH, Clp ATPases are involved in the biogenesis and degradation of proteins, including membrane-bound proteins and OMPs (Frees *et al*., 2004). Frees *et al*. (2004) have suggested that the regulation of surface-associated adhesion proteins was a likely explanation for Clp ATPase function in *S. aureus* biofilm formation. Similarly, through the regulation of surface-associated adhesion proteins, FtsH may play an important role in *F. johnsoniae* attachment and/or biofilm formation and the cell envelope integrity of the strong biofilm phenotype associated with isolate YO64.

Protein YO64_15, involved in metabolic processes, displayed significant homology to malate dehydrogenase (Tables 3.6 and 3.7), an enzyme of the tricarboxylic acid cycle, catalyzing the reversible conversion of L-malate and oxaloacetate. Trémoulet *et al*. (2002) observed increased expression of this enzyme in an *E. coli* O157:H7 biofilm and suggested that the culture method affected the central metabolism of *E. coli*. Similarly, agar surface-associated growth may affect the central metabolism of *F. johnsoniae*-like isolates.

In the present study, a shared proteome with differences in expression levels, as well as expression of unique proteins was observed for agar surface-associated, biofilm and planktonic cells of *F. johnsoniae*-like isolates YO12 and YO64, respectively. The function of putative agar surface-associated proteins more closely resembled those of the biofilm phase of *F. johnsoniae*-like isolate YO64. For isolate YO12, the agar surface-associated proteome appeared similar to that of the planktonic phase.

Neither the biofilm nor the agar surface-associated proteomes represented a mixture of planktonic cells associated with the stationary phase. Differences in protein expression between the two variations of sessile growth and that of planktonic growth of these *F. johnsoniae*-like isolates, respectively, indicate distinct physiological communities with variation in metabolic states and possible variation in coordinated cellular behavioural patterns.

The genome of *F. johnsoniae* UW101 (McBride *et al*., 2009) harbours a large number of genes encoding hypothetical proteins with unknown function, which could explain the high frequency of hypothetical (12%) and unidentifiable (26%) proteins (Tables 3.4 and 3.6) observed in the 42 proteins selected for MS in this study. Unidentifiable proteins of *F. johnsoniae*-like isolates may indicate novel proteins with no similarity to protein sequences in the current MSDB protein databases. However, it may also indicate poor mass spectra due to either contamination or poor protein quality, resulting in no identification. Subsequent repetitive MS data may be needed to confirm this and amino acid sequence determination may reveal possible functional properties of these proteins.

Interestingly none of the protein spectra displayed homology to the *F. johnsoniae* UW101 or *F. psychrophilum* motility proteins, nor were any quorum sensing molecules or regulatory system components associated with *F. johnsoniae* UW101 identified. *F. johnsoniae* expression regulatory systems include sigma factors, such as the RpoD homologue, and cyclic-nucleotide-binding proteins, possibly cAMP (McBride *et al*., 2009). Besides the identification of a putative uridylate kinase, involved in uracil, UMP and UTP regulatory

systems, in the biofilm phase of isolate YO12, no other components of signal transduction regulatory systems were identified in this study. The lack of proteins classified in these categories could be explained by spot selection, since only a limited number of protein spots were chosen for MS.

In the present study, it was demonstrated that *F. johnsoniae*-like isolates grown in a sessile state exhibit proteomes (WCPs and OMPs) that differ to various degrees from that of their planktonic counterparts. Several proteins unique to each of the planktonic, biofilm and agar surface-associated growth phases were observed for both *F. johnsoniae*-like isolates investigated in the present study. However, similar to the findings of Vilain and Brözel (2006) for *B. cereus*, the majority of the protein spots appeared to be differentially expressed.

Qualitative analysis of 2-D WCP maps provided evidence of protein induction and suppression within growth-mode specific proteomes of these *F. johnsoniae*-like isolates. Hence, the 2-D/MS proteomic approach was used successfully to demonstrate differential protein expression between planktonic, biofilm and agar surface-associated *F. johnsoniae*-like cells, and to highlight proteins unique to each of the three different growth phases, as part of an adaptive response. Although several characteristic trends were observed in the proteome of planktonic, biofilm and agar surface-associated *F. johnsoniae*-like cells, respectively, the limited number of protein spots identified using 2-D analysis and MS provided insufficient information to offer a holistic view on the physiological and protein expression changes that *F. johnsoniae*-like isolates undergo during the switch from planktonic to a sessile form of growth.

This is the first study to report the differences in protein expression between the planktonic and sessile growth phases of *F. johnsoniae*-like isolates. The proteomics approach used in the present study did not distinguish fundamental biofilm proteins from negligible proteins and the effect of altered expression of these proteins on the physiological and biochemical nature of *F. johnsoniae*-like cells. Future inactivation/expression studies may verify the role of putative proteins that play a fundamental role in the biofilm phenotype of *F. johnsoniae*-like isolates. Quantitative analysis of protein expression can be

performed by real time reverse transcription PCR to validate the expression level changes in a selection of biofilm growth-related proteins.

A more detailed expression study can be performed using a transcriptomic approach, such as SSH, to elucidate additional factors involved in the adaptive responses that the *F. johnsoniae*-like isolates undergo during the switch from planktonic to a biofilm mode of growth. Transcriptional profiling was performed using SSH in order to provide a comparison of differential gene expression between the planktonic and sessile growth phases of the *F. johnsoniae*-like isolates YO12 and YO64 to differential protein expression by 2-D gel electrophoresis and MALDI-TOF MS identification.

CHAPTER FOUR

CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED *F. johnsoniae*-like GENES OF PLANKTONIC AND SURFACE-ASSOCIATED PHASES USING SUPRESSION SUBTRACTIVE HYBRIDIZATION.

4.1. INTRODUCTION

Biofilms represent a unique mode of growth which is distinct from the free living, planktonic growth phase previously considered to be equivalent to surface-associated growth (Costerton *et al*., 1987). Bacteria existing in the biofilm mode show altered gene expression compared to their planktonic counterparts (Jefferson, 2004). Differences in the type and level of gene expression in a defined growth phase determine the phenotypic characteristics associated with that specific phase (Parkins *et al*., 2001).

The use of 2-D gel electrophoresis for investigating the differences in protein expression between planktonic and biofilm growth of a bacterium has certain limitations, i.e., the limited pI range selected for protein analysis, 2-D gel resolution and the physicochemical properties of proteins (Graves and Haystead, 200). For instance, proteins with low expression levels and/or proteins of hydrophobic nature may not be detected using 2-D gel electrophoresis. Although capable of resolving a large part of the proteome, a certain percentage of proteins, including over-expressed and uniquely expressed proteins, may not be identified (Graves and Haystead, 200).

Transcriptomic techniques, on the other hand, have the ability to identify most up-regulated or uniquely expressed genes, including those expressed at low levels, i.e., rare transcripts (Parkins *et al*., 2001). Using DNA microarray analysis, Whiteley *et al*. (2001) demonstrated that only a small percentage (1%) of *Pseudomonas aeruginosa* genes were subject to differential expression responsible for the shift between the planktonic and biofilm lifestyles. Additionally, biofilm-regulated genes also indicated some of the mechanisms of

antimicrobial resistance observed in the biofilm mode, which included the up-regulation of putative efflux systems (Whiteley *et al*., 2001). In *Streptococcus mutans*, a similar trend was observed, where only a small number of genes displayed differential expression during the transition from the free-living to biofilm states, resulting in significant physiological change to support the biofilm phenotype (Shemesh *et al*., 2007). Of the genes investigated, 57% were up-regulated and 43% were down-regulated in *S. mutans* biofilm cells (Shemesh *et al*., 2007). Significantly up-regulated genes were involved in stress response, such as SOD; membrane/transport, including ABC transporters; and metabolic processes, including amino acid and carbohydrate metabolism (Shemesh *et al*., 2007). Conversely, Sauer *et al*. (2002) demonstrated a large physiological change, where more than 800 gene products with a 6-fold change or greater in expression, occurred in biofilm development of *P. aeruginosa* compared to the planktonic phase.

However, most transcriptomic techniques have the disadvantage that large quantities of mRNA and/or whole genome arrays are required (Becker *et al*., 2001). To address these problems, cDNA subtraction techniques, such as SSH and micro-representational-difference analysis (Becker *et al*., 2001), which require very little starting RNA material have been developed. Subtractive hybridization is widely used for comparison of closely related organisms at the genomic level, distinguishing between virulent and avirulent bacteria at the genetic level and for identifying genes uniquely expressed in a defined growth phase (Cummings *et al*., 2004; Marenda *et al*., 2005; Nesbø *et al*., 2002; Olivares-Fuster and Arias, 2008; Parkins *et al*., 2001; Qi *et al*., 2005; Saxena *et al*., 2005; Soule *et al*., 2005; and Winstanley, 2002). SSH has also been used successfully in the identification of genomic diversity between related organisms and virulence determinants in pathogenic strains of bacteria (Winstanley, 2002). Recently, genetic differences between *Flavobacterium columnare* and *Flavobacterium johnsoniae* (Olivares-Fuster and Arias, 2008), and two *Flavobacterium psychrophilum* strains (Soule *et al*., 2005), respectively, were successfully assessed with SSH.

The identification of differentially expressed genes between independent growth phases of bacteria by SSH is relatively new (Parkins *et al*., 2001). The modified SSH technique enables researchers to target uniquely expressed and over-expressed genes from a defined growth phase, such as biofilm growth. Unlike traditional genetic techniques, such as transposon-mediated mutagenesis, SSH has the potential for identifying all factors differentially expressed in a defined growth phase (Parkins *et al*., 2001). Sauer and Camper (2001) successfully used SSH to demonstrate the differential expression of 40 genes in surface-associated *Pseudomonas putida* cells. Twenty-eight of the genes displayed homology to known proteins (Sauer and Camper, 2001). The NlpD lipoprotein and proteins involved in amino acid metabolism were shown to be down-regulated, while components of ABC transporters, structural components of type IV pili, and proteins involved in polysaccharide biosynthesis were up-regulated (Sauer and Camper, 2001).

Protein profiling, using both 1-D and 2-D gel electrophoresis (Chapter 3) revealed differences in *F. johnsoniae*-like isolates proteomes when planktonic, biofilm and agar surface-associated cells, respectively, were compared. However, limitations observed included unidentifiable proteins and difficulty in quantitative comparison due to wide variation in proteomes at different growth phases. Ideally, phenotypic and genetic differences between bacteria in the respective planktonic and biofilm states should be examined by comparative analysis in order to identify those genes specifically involved in or required for biofilm formation. Therefore, a polyphasic approach involving proteomic investigations as well as transcriptional profiling is recommended for molecular biofilm research (Turner *et al*., 2005). The wealth of sequence data and proteomic databases currently available online facilitates comparative analysis of molecular research at both the genetic and proteomics level.

In the present study, SSH was used for the identification of target cDNAs that correspond to rare transcripts uniquely expressed or over-expressed in the biofilm and agar surface-associated phases of *F. johnsoniae*-like isolates YO12 and YO64, respectively. Biofilm and agar surface-associated cells were used as

tester populations, respectively, and cDNA copies of RNA extracted from these two populations were compared to cDNA copies of RNA extracted from planktonic cells (driver population).

4.2. MATERIALS AND METHODS

4.2.1. Growth of *F. johnsoniae* Cultures

F. johnsoniae-like isolates YO12 and YO64 used for proteomic profiling were also selected for transcriptional profiling using SSH of planktonic and biofilm, and planktonic and agar surface-associated growth, respectively. Planktonic, biofilm and agar surface-associated cells were grown as described in Chapter 3, section 3.2.1.

4.2.2. Total RNA Extraction

Total cellular RNA was extracted from planktonic, biofilm and agar surface-associated cells by a rapid isolation technique described for Gram-negative bacteria (Reddy and Gilman, 1993). Cells were harvested by centrifugation at 10 000 rpm and cell pellets were resuspended in 10 ml protoplasting buffer [15 mM Tris.Cl (pH 8.0), 0.45 M sucrose, and 8 mM EDTA). Eighty µl of 50 mg/ml lysozyme was added and samples were incubated for 15 min on ice. Protoplasts were collected by centrifugation at 7000 rpm for 5 min and protoplast pellets were resuspended in 0.5 ml Gram-negative lysing buffer [10 mM Tris.Cl (pH 8.0), 10 mM NaCl, 1 mM Na-citrate, and 1.5% SDS]. Fifteen µl of diethylpyrocarbonate (DEPC) was added and samples were mixed gently before the contents were transferred to microcentrifuge tubes and incubated for 5 min at 37°C. After tubes were chilled on ice, 250 µl of saturated NaCl (40 g NaCl in 100 ml ddH_2O) was added and samples were mixed by inversion. After a further

incubation for 10 min on ice, tubes were centrifuged at 13 000 rpm for 10 min. Supernatants were transferred to two clean microcentrifuge tubes and 1 ml ethanol was added. RNA was precipitated O/N at -20°C. After precipitation, tubes were centrifuged for 15 min at 4°C. Resulting pellets were rinsed and left to dry. Dried pellets were resuspended in 100 µl DEPC-treated ddH_2O and stored at -70°C.

4.2.3. mRNA Isolation from Total RNA

mRNA isolation was performed with the Oligotex mRNA purification kit using the Oligotex mRNA Spin-Column Protocol (Qiagen, USA), according to the manufacturer's instructions. Total RNA samples were adjusted to 250 µl with RNase-free water and 250 µl of Buffer OBB and 15 µl of Oligotex Suspension was added. Contents were thoroughly mixed by pipetting and incubated at 70°C for 3 min in a heating block. Samples were then removed from the heating block and incubated at 30°C for 10 min. The Oligotex/mRNA complex was centrifuged for 2 min at maximum speed and supernatants were carefully removed by pipetting. Pellets were resuspended in 400 µl Buffer OW2 by pipetting and transferred to a small spin column. Spin columns were centrifuged for 1 min at maximum speed and placed in a clean microcentrifuge tube. Four hundred µl Buffer OW2 was added to each column and columns were centrifuged for 1 min at maximum speed. Spin columns were transferred to a clean microcentrifuge tube and 50 µl hot (70°C) Buffer OEB was added directly to each column and pipetted up and down 4 times. Columns were centrifuged for 2 min at maximum speed and mRNA samples were stored at -70°C until further use. RNA purity and concentration was determined spectrophotometrically using the NanoDrop™ (NanoDrop Technologies™, USA).

4.2.4. SSH Protocol

The PCR-Select™ cDNA subtraction kit (Clontech, USA) was used for SSH, according to the manufacturer's instructions. The GeneAmp PCR System 9700 thermal cycler (Perkin Elmer Applied Biosystems, USA) was used for all incubation steps (unless indicated otherwise) and PCR reactions.

4.2.4.1. First-strand cDNA synthesis

To generate first-strand cDNA from each tester and driver mRNA samples, as well as the control poly A^+ RNA, the following reagents were combined in a 0.5 ml microcentrifuge tube: 4 µl mRNA (2 µg) (2 µl control poly A^+ RNA) and 1 µl cDNA synthesis primer (10 µM). Contents were mixed thoroughly and incubated at 70°C for 2 min in the GeneAmp PCR System 9700 thermal cycler. Tubes were cooled on ice for 2 min, briefly centrifuged and the following reagents added: 2 µl 5× first-strand buffer [250 mM Tris.HCl (pH 8.5), 40 mM $MgCl_2$, 150 mM KCl, and 5 mM DTT], 1 µl dNTP mix (10 mM each), 1 µl sterile H_2O and 1 µl AMV reverse transcriptase (20 units/µl). Contents were gently vortexed and briefly centrifuged before incubation at 42°C for 1.5 h in an air incubator. First-strand cDNA synthesis was terminated by placing the tubes on ice and second-strand cDNA synthesis was commenced immediately.

4.2.4.2. Second-strand cDNA synthesis

The following components were added to the first-strand tester, driver and control cDNAs: 48.4 µl sterile H_2O, 16 µl 5× second-strand buffer [500 mM KCL, 50 mM NH_3SO_4, 25 mM $MgCl_2$, 0.75 mM β-NAD, 100 mM Tris.HCl (pH 7.5), and 0.25 mg/ml BSA], dNTP mix (10 mM), and 20× second-strand enzyme cocktail (6 U/µl

DNA polymerase I, 0.25 U/µl RNase H, and 1.2 U/µl *E. coli* DNA ligase). Contents were briefly centrifuged before incubation at 16°C for 2 h in a thermal cycler. Two µl T4 DNA polymerase was added and mixed well before incubation at 16°C for 30 min in a thermal cycler. Four µl 20× EDTA/glycogen mix (0.2 M EDTA, and 1 mg/ml glycogen) was added to terminate second-strand synthesis.

One hundred µl phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed thoroughly and centrifuged at 14 000 rpm for 10 min at RT. The upper layer was placed in a clean tube and 100 µl chloroform:isoamyl alcohol (24:1) was added. Tubes were vortexed thoroughly and centrifuged at 14 000 rpm for 10 min at RT. The upper layer was placed in a clean tube and 40 µl 4 M CH_3COONH_4 and 300 µl 95% ethanol was added. Tubes were vortexed thoroughly and centrifuged at 14 000 rpm for 20 min at RT. Supernatants were carefully removed and pellets were overlaid with 500 µl 80% ethanol and centrifuged at 14 000 rpm for 10 min at RT. Supernatants were carefully removed and pellets were air-dried and dissolved in 50 µl sterile H_2O and stored at -20°C until *Rsa*I digestion.

4.2.4.3. *Rsa*I digestion

In order to create smaller blunt-end tester and driver cDNA fragments, the generated cDNAs were digested with *Rsa*I. The following reagents were added to 43.5 µl of each tester, driver and control second-strand cDNAs: 5 µl 10× *Rsa*I restriction buffer [100 mM Bis Tris propane-HCl (pH 7.0), 100 mM $MgCl_2$, and 1 mM DTT] and 1.5 µl *Rsa*I (10 U/µl). Tubes were mixed by vortexing and briefly centrifuged before incubation at 37°C for 1.5 h. Two and a half µl of 20× EDTA/glycogen mix (0.2 M EDTA, and 1 mg/ml glycogen) was added to terminate second-strand synthesis. Fifty µl phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed thoroughly and centrifuged at 14 000 rpm for 10 min at RT. The upper layer was placed in a clean tube and 50 µl

chloroform:isoamyl alcohol (24:1) was added. Tubes were vortexed thoroughly and centrifuged at 14 000 rpm for 10 min at RT. The upper layer was placed in a clean tube and 25 µl 4 M NH_4OAc and 187.5 µl 95% ethanol were added. Tubes were vortexed thoroughly and centrifuged at 14 000 rpm for 20 min at RT. Supernatants were removed and pellets were overlaid with 200 µl 80% ethanol and centrifuged at 14 000 rpm for 5 min at RT. Supernatants were carefully removed and pellets were air dried and dissolved in 5.5 µl sterile H_2O and stored at -20°C. These samples served as experimental driver and control driver cDNAs.

4.2.4.4. Adaptor ligation

Tester and control tester cDNAs were ligated with adaptors for forward and control reactions. One µl *Rsa*I-digested experimental tester cDNA was diluted with 5 µl sterile H_2O and control cDNA was diluted with diluted ϕX174/*Hae*III control DNA according to the manual instructions, prior to adaptor ligation.

A ligation master mix was prepared by combining the following reagents: 3 µl sterile H_2O, 2 µl 5× ligation buffer [250 mM Tris.HCl (pH 7.8), 50 mM $MgCl_2$, 10 mM DTT, and 0.25 mg/ml BSA] and 1 µl T4 DNA ligase (400 U/µl). Ligation reagents for each adaptor were added to a 0.5 ml microcentrifuge tube in the following order: 2 µl diluted tester cDNA, 2 µl of adaptors 1 or 2R (10 µM), respectively, and 6 µl master mix. Contents were mixed thoroughly by pipetting. Two µl of each tester-adaptor mixture was mixed in a clean microcentrifuge tube to serve as the unsubtracted tester control. Tubes were briefly centrifuged and incubated O/N at 16°C. One µl EDTA/glycogen mix was added to terminate the ligation reaction and samples were heated at 72°C for 5 min to inactivate the ligase.

One µl of the completed unsubtracted tester control was removed and diluted in 1 ml sterile H_2O for PCR analysis. All samples were stored at -20°C.

Ligation efficiency analysis of the control cDNA was performed prior to the hybridization steps as described in the PCR-Select™ cDNA subtraction manual.

4.2.4.5. First hybridization

In the first hybridization, excess driver cDNA was added to each adapter-ligated tester cDNA to enrich for differentially expressed sequences. Hybridization reagents were combined in 0.5 ml microcentrifuge tubes for each experimental and control subtractions in the following order: 1.5 µl *Rsa*I-digested driver cDNA, 1.5 µl adaptor 1-ligated or adaptor 2R-ligated tester, respectively, and 1 µl 4× hybridization buffer. Samples were overlaid with one drop mineral oil and centrifuged briefly before incubation at 98°C for 1.5 min in the thermal cycler. Samples were then incubated at 68°C for 8 h.

4.2.4.6. Second hybridization

The two samples from the first hybridization were mixed and freshly denatured driver cDNA was added to further enrich for differentially expressed sequences. Hybridization reagents were combined in 0.5 ml microcentrifuge tubes: 1 µl driver cDNA, 1 µl 4× hybridization buffer, and 2 µl sterile H_2O. For each experimental and control tester cDNA, 1 µl of this mixture was placed in a clean 0.5 ml microcentrifuge tube, overlaid with mineral oil and incubated at 98°C for 1.5 min in a thermal cycler. Freshly denatured driver cDNA was then mixed with the two hybridization samples from the first hybridization according to the manufacturer's instructions. Contents were briefly centrifuged and incubated O/N at 68°C. Two hundred µl dilution buffer [20 mM HEPES (pH 6.6), 20 mM NaCl, and 0.2 mM EDTA (pH 8.0)] was added and mixed by pipetting before incubation at 68°C for 7 min in a thermal cycler. All samples were stored at -20°C until further use.

Subtraction efficiency was determined by using PCR analysis with G3PDH primers according to the manufacturer's instructions.

4.2.4.7. PCR amplification

Differentially expressed cDNAs were selectively amplified during the two PCR reactions as described below. One µl of each diluted cDNA (each subtracted sample and the corresponding unsubtracted tester control) and control subtracted cDNA were aliquoted into PCR tubes and the following PCR reagents were added to each tube: 19.5 µl sterile H_2O, 2.5 µl 10× PCR reaction buffer, 0.5 µl dNTP mix (10 mM), 1 µl PCR primer 1 (10 µM), and 0.5 µl 50× Advantage™ cDNA Polymerase Mix (Clontech). Contents were mixed well by vortexing, briefly centrifuged, overlaid with mineral oil and incubated at 75°C for 5 min in the thermal cycler. Primary PCR was commenced using the following cycling parameters: 94°C for 25 s, followed by 27 amplification cycles of 94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min. Eight µl from each tube was analyzed on 2% agarose/ethidium bromide gel run in 1× TAE buffer.

Three µl of each primary PCR mixture was then diluted in 27 µl sterile H_2O of which 1 µl was aliquoted into clean PCR tubes for secondary PCR. The following PCR reagents were added to each tube: 18.5 µl sterile H_2O, 2.5 µl 10× PCR reaction buffer, 1 µl nested PCR primer 1 (10 µM), 1 µl nested PCR primer 2R (10 µM), 0.5 µl dNTP mix (10 mM), and 0.5 µl 50× Advantage™ cDNA Polymerase Mix (Clontech). The contents were mixed well by vortexing and briefly centrifuged. Samples were overlaid with one drop mineral oil and secondary PCR was commenced using the following cycling parameters: 12 cycles of 94°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min. Eight µl from each tube was analyzed on 2% agarose/ethidium bromide gel run in 1X TAE buffer.

Visual analysis of secondary PCR products revealed poor amplification. Thus, the protocol was modified in order to obtain optimal amounts of primary

PCR products. This modification involved increasing the number of amplification cycles for the primary PCR from 27 to 30. The resulting amplicons were used for further analysis. All reaction products were stored at -20°C until further use.

4.2.5. Cloning of PCR Products in *E. coli* DH5α

4.2.5.1. Ligation

The pGEM-T Easy vector system (Promega) was used to ligate purified PCR products. Reactions were carried out in 10 µl reaction volumes containing 3 µl insert DNA (10-50 ng), 1 µl pGEM-T Easy Vector (50 ng/µl), 5 µl 1× rapid ligation buffer [30 mM Tris.HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 5% (w/v) polyethylene glycol MW 8000] and 1 µl T4 DNA ligase (3 Weiss units). Samples were incubated at 4°C for 16 h and stored at -20°C.

4.2.5.2. Electrotransformation of *E. coli*

Electrocompetent *E. coli* DH5α cells were used for high efficiency electro-transformation. Fresh O/N *E. coli* culture was used to inoculate 200 ml LB flasks (1% v/v) and incubated at 37°C until the cell density was equivalent to 0.5 at OD_{600}. The cell suspension was centrifuged at 4000× g for 10 min at 4°C. The pellet was washed twice in 100 ml cold water by centrifugation and the supernatant removed. Cells were resuspended in 20 ml cold 10% glycerol and centrifuged at 4000× g for 10 min at 4°C. Cells were resuspended in a final volume of 2 ml cold 10% glycerol and 40 µl aliquots were stored at -70°C.

Electrotransformation of electrocompetent cells was performed in a Bio-Rad Gene Pulser (Bio-Rad). Briefly, 5 µl of ligation mixture was added to 40 µl of competent cells, mixed well and placed on ice for 1 min. The mixture was then

transferred to a cold electroporation cuvette and electroporated in a Bio-Rad Gene Pulser at 2.5 kV (25 μF; 300 Ω). The cuvette was removed immediately after electroporation and 1 ml SOC medium [2% tryptone (w/v), 0.5% yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose] was added. The cell suspension was mixed by pipetting and transferred to a 1 ml centrifuge tube and incubated at 37°C for 1 h. One-hundred microliters of the cell suspension was spread-plated onto LB plates containing ampicillin (100 μg/ml final conc.) and incubated O/N at 37°C (Sambrook *et al*., 1989). Two hundred and fifty clones (120 of isolate YO12 and 130 of isolate YO64) were selected and duplicate freeze stocks were prepared in freezing medium and stored at -70°C.

4.2.6. Sequencing

Initially, 10 clones were selected for sequencing based on insert sizes. Once sequenced, these inserts were used as probes in Southern dot blots to screen the cDNA-clone library to eliminate possible duplicate sequencing. A total of 32 clones were selected for sequencing. DNA inserts were sequenced using the ABI373A automatic sequencer (Perkin Elmer Applied Biosystems).

DNA sequences were analyzed, edited and assembled in DNAMAN (version 4.0, Lynnon BioSoft, Canada). DNA sequence similarity searches were performed using the BLAST 2.9 algorithm (Altschul *et al*., 1997). Sequences were identified using the nucleotide, translated nucleotide and protein databases. Alignments of DNA sequences to reference sequences, obtained from GenBank, were performed using DNAMAN.

4.2.7. Southern Dot Blot Analysis for Clone Screening

To eliminate multiple copies of a single clone in the cDNA clone library, Southern dot blot analysis was performed using the DIG-system (Roche, Germany). One µl denatured plasmid DNA from each of the 250 selected clones were spotted on a nylon Hybond-N+ membrane (Amersham, UK) and allowed to air dry. DNA was crosslinked under UV light for 3 min.

Plasmid DNA of 10 selected clones (YO12_B4, YO12_B7, YO12_A24, YO12_A31, YO12_A52, YO64_B6, YO64_B23, YO64_B49, YO64_A2 and YO64_A13,) were isolated by the rapid plasmid isolation technique (Birnboim, 1983). cDNA inserts were excised from the pGEM-T easy vector using *Eco*RI. Fragments were subjected to electrophoresis in 1.5% agarose/ethidium bromide gel run in 1× TAE buffer. Inserts were excised from gels and extracted from the agarose using the QIAquick gel extraction kit (Qiagen). Thereafter, inserts were labeled using the DIG high prime DNA-labeling kit (Roche).

Ten µl of insert DNA was denatured by boiling for 10 min and snap-cooled on ice+EtOH. The following reagents for random primed DIG-labeling were added on ice: 2 µl 10× hexanucleotide primer mix, 2 µl 10× dNTPs labeling mix, 5 µl dd H_2O, and 1 µl Klenow (1 U/µl). Contents were incubated at 37°C for 30 min after which the reaction was stopped by boiling for 10 min. DIG-labeled probes were stored at -20°C until further use.

The plasmid DNA-containing membrane was pre-hybridized in DIG-EASY Hyb for 2 h at 46°C. DIG-labeled probes were boiled for 10 min and snap-cooled on ice+EtOH. Denatured probes were added to the hybridization buffer and hybridized O/N at 46°C, respectively.

After hybridization, stringency washes were performed to remove non-specifically bound and unbound probe. The membrane was washed twice in 2× SSC/0.1% SDS [10 ml 20× SSC (3 M NaCl, 75 mM sodium citrate, pH 7.0), 1 ml 10% SDS, ddH_2O to 100 ml] for 15 min at RT. The membrane was then washed

twice in 0.1× SSC/0.1% SDS (0.5 ml 20× SSC, 1 ml 10% SDS, ddH_2O to 100 ml) for 20 min at 65°C.

Bound probe was detected using chemiluminescence according to the DIG-system detection protocol. The membrane was washed for 5 min in Wash Buffer [1 ml Tween 20 and 199 ml Buffer 1 (0.1 M Maleic acid, 0.15 M NaCl, ddH_2O to 1 l)] at RT. Equilibration of the membrane was performed in 10 ml Blocking solution for 30 min. The membrane was then incubated in Anti-DIG Fab fragment/Blocking solution for 30 min and washed twice in 100 ml Wash Buffer for 15 min. After briefly soaking the membrane in Detection Buffer [100 mM Tris.HCl (pH 9.5), and 100 mM NaCl], the membrane was placed in a plastic bag and 1 ml CSPD was added directly to the membrane surface. The membrane was incubated at 37°C for 15 min and exposed to X-ray film in a dark room.

For re-probing, the membrane was stripped by incubation in 0.2 N NaOH at 37°C for 30 min, and agitation with boiling 0.1% SDS, three times for 5 min, respectively. The membrane was pre-hybridized and hybridization with the next probe was performed as before.

4.2.8. Virtual Northen Blot Analysis

The expression patterns of 10 randomly selected inserts, five from each isolate, i.e., YO12 and YO64, were analyzed using virtual Northern blot analysis (Endege *et al*., 1999). Approximately 2 μg of cDNA generated from the total RNA samples of isolates YO12 and YO64 grown planktonically, as a biofilm and as agar surface-associated growth, respectively, were transferred to a nylon membrane using a slot blot manifold. Inserts from 10 selected clones, YO12_B4, YO12_B7, YO12_A24, YO12_A31, YO12_A52, YO64_B6, YO64_B23, YO64_B49, YO64_A2 and YO64_A13, used previously in Southern hybridization, were used as probes. These had been prepared as described in section 4.2.7. Genomic DNA from isolates YO12 and YO64 was used as positive controls and DIG-

labeled amplicons of the housekeeping gene EF-TU (elongation factor TU) were used as positive expression control for each isolate, respectively.

Quantification of differential gene expression was performed by integrated density value analysis of the positive bands using Alpha Imaging software (Alphainnotech, USA). Gene expression levels were compared to that of EF-TU and the integrated density values of the genomic DNA bands were used as reference for standardization.

4.3. RESULTS

4.3.1. PCR Analysis of Subtraction Efficiency

PCR was used to determine subtraction efficiency by comparing the abundance of cDNA before and after subtraction. G3PDH reduction (Fig. 4.1, lanes 11-14) demonstrated successful control subtraction of the control cDNA prepared during the experimental procedure. The PCR control-subtracted cDNA (Clontech) (Fig.4.1, lanes 2-5) demonstrated little to no G3PDH reduction compared to the experimental subtracted control.

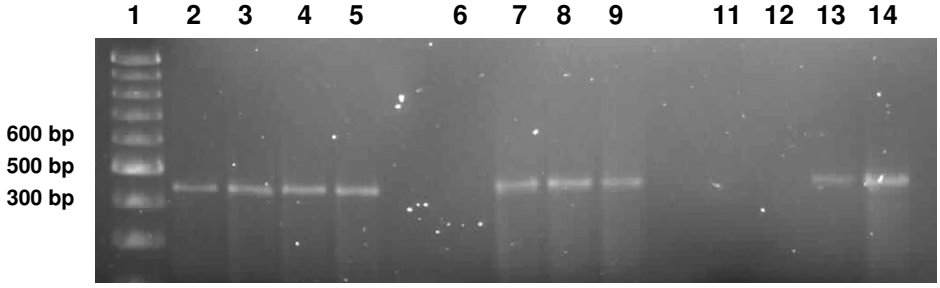

FIG. 4.1. Agarose gel electrophoresis of subtraction efficiency-PCR fragments using G3PDH primers. Lane 1: GeneRuler 100 bp Plus DNA ladder (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp; Fermentas, Canada); lanes 2-5: PCR control subtracted cDNA; lanes 6-9: unsubtracted experimental control cDNA; and lanes 11-14: successfully subtracted experimental control cDNA.

4.3.2. Primary PCR Products

The primary PCR products of the experimental control-subtracted cDNA corresponded to ϕX174/*Hae*III-digested DNA fragment sizes (1300/1100/900/600/300/200 bp) (Fig. 4.2, lane 3). The adaptor sequences on both ends of DNA fragments caused a slight mobility shift of these PCR fragments.

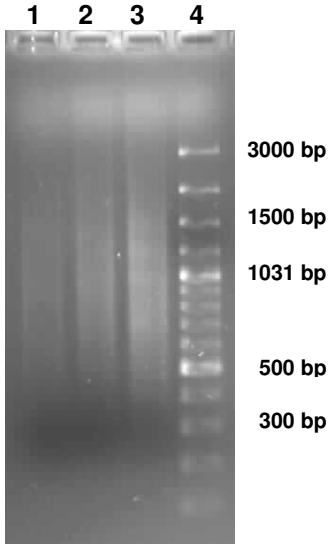

FIG. 4.2. Agarose gel electrophoresis of primary PCR fragments following subtraction. Lane 1: YO12 agar surface-attached subtracted cDNA amplicons; lane 2: YO12 biofilm subtracted cDNA amplicons; lane 3: experimental control cDNA amplicons; and lane 4: GeneRuler 100 bp Plus DNA ladder (3000/2000/1500/ 1200/1031/900/800/700/600/500/400/300/200/100 bp; Fermentas, Canada).

4.3.3. Clone Selection

pGEM-T vectors containing the *F. johnsoniae*-like isolates YO12 and YO64 inserts ranged from 2.69 kb to 4.25 kb in molecular weight (Fig. 4.3). Clones containing plasmids with inserts ranging between 0.3 kb – 2.0 kb were selected for sequencing.

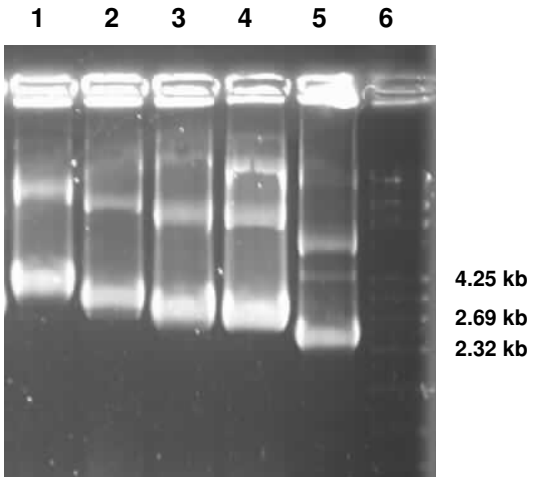

FIG. 4.3. Agarose gel electrophoresis of plasmid DNA from *F. johnsoniae*-like isolate YO64 clones displaying different size inserts. Lanes 1-4: Clones YO64_B49 – YO64_B52, respectively; lane 5: pGEM-T without insert; and lane 6: Marker IV (19.32/7.74/5.53/4.25/3.14/2.69/2.32/1.88/1.49/1.15/0.93/0.69/0.42 kb; Roche, Germany).

Insert probes of the 10 selected clones, YO12_B4, YO12_B7, YO12_A24, YO12_A31, YO12_A52, YO64_B6, YO64_B23, YO64_B49, YO64_A2 and YO64_A13 of isolates YO12 and YO64, yielded varying Southern dot blot hybridization results, ranging from unique to multiple signals. Fifty-four of the 120 selected clones of isolate YO12 displayed positive signals with the five YO12 inserts: clone YO12_B4 insert displayed 23 positive signals, clone YO12_B7 insert displayed 3, clone YO12_A24 insert displayed 5, clone YO12_A31 insert displayed 18, and clone YO12_A52 insert displayed 4. While 48 of the 130 selected clones of isolate YO64 displayed positive signals with the five YO64 inserts: clone YO64_B6 insert displayed 1 positive signal, clone YO64_B23 insert displayed 8, clone YO64_B49 insert displayed 10, clone YO64_A2 insert displayed 20, and clone YO64_A13 insert displayed 9. Clones displaying a positive signal with any of the probes were eliminated to prevent repetitive sequencing of identical inserts.

4.3.4. Sequence Analysis

In total, 16 clones from isolate YO12 and 16 clones from isolate YO64 were selected for gene sequence analysis (Tables 4.1 and 4.2). BLAST results indicated that 60% and 81% of the 16 selected clones from isolates YO12 and YO64, respectively, contained inserts that shared sequence identity to genes of *F. johnsoniae* UW101. Majority of the insert sequences displayed identity to known genes within functional categories of metabolic processes, transcription/translation, adaptation/protection, membrane proteins/transport/ binding proteins/motility, with only a single insert displaying homology to a hypothetical protein (Tables 4.1 and 4.2).

Of the six YO12 agar surface-associated clone inserts sequenced and identified, 67% (4/6) were categorized as being involved in membrane/transport/ motility, while 33% (2/6) were involved in metabolic processes (Table 4.1). A wider variety was observed for YO12 biofilm clones, with 40% (4/10) of the inserts being associated with membrane proteins/transport/motility, 20% (2/10) with metabolic processes, 40% (4/10) with adaptation/protection, and 20% (2/10) were with transcription/translation (Table 4.1).

Of the nine YO64 biofilm clone inserts sequenced and identified, 44% (4/9) were categorized as being associated with adaptation/protection, 22% (2/9) with metabolic processes, and 22% (2/9) with membrane/transport/motility (Table 4.2). The seven YO64 agar surface-associated clones were assigned to similar functional categories, with 43% (3/7) being associated with adaptation/protection, 29% (2/7) with metabolic processes, and 29% (2/7) with membrane/transport/ motility (Table 4.2).

4.3.5. Expression Patterns

All the cloned insert gene sequences used as probes displayed differential expression patterns (Figs. 4.4 and 4.5). Quantification of expression levels by

TABLE 4.1 Identification and functional categorization of differentially expressed *F. johnsoniae*-like isolate YO12 genes in the biofilm and agar surface-associated phases by SSH

Growth phase	Clone no.	Length (bp)	Identified Protein (Gene name)*	Organism	Identity %	Functional category	No. of matched peptides
Biofilm	YO12_B2	566	AsnC family transcriptional regulator (*asnC*; Fjoh_2805)	*Flavobacterium johnsoniae*	97%	Transcription/translation	>100
	YO12_B4	356	Major facilitator superfamily (MFS) transporter (OM2255_14645)	alpha proteobacterium	37%	Membrane/transport/motility	1
	YO12_B7	652	MATE efflux family protein (*matE*; Fjoh_3175)	*Flavobacterium johnsoniae*	55%	Membrane/transport/motility	>100
	YO12_B11	659	TRAP-type transporter (HRM2_16140)	*Desulfobacterium autotrophicum*	27%	Membrane/transport/motility	1
	YO12_B9	559	Ribonuclease H (*rnh*; Fjoh_1135)	*Flavobacterium johnsoniae*	91%	Transcription/translation	>100
	YO12_B30	772	Glutamate synthase, NADH/NADPH subunit (*gltB*; Fjoh_2160)	*Flavobacterium johnsoniae*	95%	Adaptation/protection	>100
	YO12_B46	807	Hypothetical protein BACCOPRO_01435 (BACCOPRO_01435)	*Bacteroides coprophilus*	47%	Hypothetical	37
			GPW/gp25 family protein (*gp25*; Fjoh_3263)	*Flavobacterium johnsoniae*	25%	Adaptation/protection	
	YO12_B52	1096	Putative chitinase (Hflu203000891)	*Haemophilus influenzae*	45%	Metabolic processes	>100
			EF hand domain-containing protein (PSPTO_5204)	*Pseudomonas syringae*	33%	Adaptation/protection	
	YO12_B58	788	Hypothetical protein (Fjoh_0539)	*Flavobacterium johnsoniae*	81%	Hypothetical	28
			Chloride channel protein (*clc*; FP1773)	*Flavobacterium psychrophilum*	33%	Membrane/transport/motility	
	YO12_B59	633	AMP-binding enzyme (*luxE*)	*Bacillus mycoides*	35%	Metabolic processes	>100
			Peptide synthetase (Avi_3342)	*Agrobacterium vitis*	31%	Adaptation/protection	
Agar	YO12_A4	465	Polysaccharide biosynthesis protein (Msp_0066)	*Methanosphaera stadtmanae*	38%	Metabolic processes	>100
	YO12_A24	781	Putative lipoprotein (CPE1647)	*Clostridium perfringens*	29%	Membrane/transport/motility	57
	YO12_A31	552	Hypothetical protein (Fjoh_2230)	*Flavobacterium johnsoniae*	54%	Unknown	39
			Efflux ABC transporter, permease protein (*ftsX*)	*Clostridium butyricum*	27%	Membrane/transport/motility	
	YO12_A32	352	RND family efflux transporter MFP subunit (*mfp*; Fjoh_1672)	*Flavobacterium johnsoniae*	90%	Membrane/transport/motility	>100
	YO12_A52	420	Putative HlyD family secretion protein (*mfp*)	*Pedobacter* spp.	60%	Membrane/transport/motility	>100
			Secretion protein HlyD (Gmet_30308)	*Geobacter metallireducens*	58%	Membrane/transport/motility	
			Efflux transporter, RND family, MFP subunit (Gbem_2203)	*Geobacter bemidjiensis*	53%	Membrane/transport/motility	
	YO12_A60	689	Delta-1-pyrroline-5-carboxylate dehydrogenase (*rocA*; Fjoh_1753)	*Flavobacterium johnsoniae*	97%	Metabolic processes	>100

* Gene name corresponding to identified protein indicated within ().

TABLE 4.2 Identification and functional categorization of differentially expressed *F. johnsoniae*-like isolate YO64 genes in the biofilm and agar surface-associated phases by SSH

Growth phase	Clone no.	Length (bp)	Identified Protein (Gene name)*	Organism	Identity %	Functional category	No. of matched peptides
Biofilm	YO64_B4	1087	Glycoside hydrolase family protein (glyco_hydro_2; Fjoh_4075)	*Flavobacterium johnsoniae*	82%	Metabolic processes	75
	YO64_B5	552	DAHP synthetase I/KDSA (*aro*; Fjoh_0514)	*Flavobacterium johnsoniae*	96%	Metabolic processes	>100
	YO64_B6	787	GumN Family protein (*gumN*; Fjoh_0812)	*Flavobacterium johnsoniae*	43%	Adaptation/protection	11
	YO64_B23	298	Hypothetical protein BACSTE_03846 (BACSTE_03846)	*Bacteroides stercoris* ATCC 43183	60%	Hypothetical	24
	YO64_B35	1146	Glutamyl-tRNA reductase (*hemA*; Fjoh_3752)	*Flavobacterium johnsoniae*	62%	Adaptation/protection	>100
	YO64_B49	1095	Hypothetical protein BACFIN_00668 (BACFIN_00668)	*Bacteroides finegoldii*	37%	Hypothetical	72
			Alginate lyase (*algL*)	*Sphingomonas* sp.	31%	Adaptation/protection	
	YO64_B60	1079	RagB/SusD domain protein (*susD*; Fjoh_0404)	*Flavobacterium johnsoniae*	39%	Membrane/transport/motility	>100
			SusD homolog (BT_0318)	*Bacteroides thetaiotaomicron*	31%		
			Putative lipoprotein (M23134_04703)	*Microscilla marina* ATCC 23134	27%		
	YO64_B68	884	NAD(P)H dehydrogenase (quinone) (*nqo1*; Fjoh_4532)	*Flavobacterium johnsoniae*	83%	Metabolic processes	>100
	YO64_B69	748	SprD (*sprD*; Fjoh_0980)	*Flavobacterium johnsoniae*	85%	Membrane/transport/motility	>100
			Putative cell surface protein precursor SprD (*sprD*)	*Flavobacterium psychrophilum*	45%		
Agar	YO64_A2	938	Ubiquinone/menaquinone biosynthesis methyltransferase (*ubiA-menA*; Fjoh_1467)	*Flavobacterium johnsoniae*	75%	Metabolic processes	>100
	YO64_A13	582	Diaminopimelate epimerase (*dap*; Fjoh_0018)	*Flavobacterium johnsoniae*	92%	Metabolic processes	>100
	YO64_A24	869	Competence/damage-inducible protein CinA (*cinA*; Fjoh_4984)	*Flavobacterium johnsoniae*	87%	Adaptation/protection	>100
	YO64_A36	1079	Cell-surface large adhesin (HS_0478)	*Haemophilus somnus*	36%	Membrane/transport/motility	>100
	YO64_A41	1053	Putative lipoprotein (Fjoh_2327)	*Flavobacterium johnsoniae*	82%	Membrane/transport/motility	>100
	YO64_A44	1076	SAM-dependent methyltransferase (*trmA;* Fjoh_4599)	*Flavobacterium johnsoniae*	80%	Adaptation/protection	>100
	YO64_A58	1093	Transketolase, central region (*tkt*; Fjoh_0049)	*Flavobacterium johnsoniae*	98%	Metabolic processes	>100

* Gene name corresponding to identified protein indicated within ().

integrated density analysis of the 10 selected YO12 and YO64 insert probes are indicated in Table 4.3. The quantified x-fold change in density values confirmed differential expression of clone YO12_A24, YO12_A31 and YO12_A52 inserts in each of the biofilm and agar surface-associated phases. Over- and/or unique expression of YO12_B4 and YO12_B7 inserts in the biofilm phase was also observed (Fig. 4.4; Table 4.3).

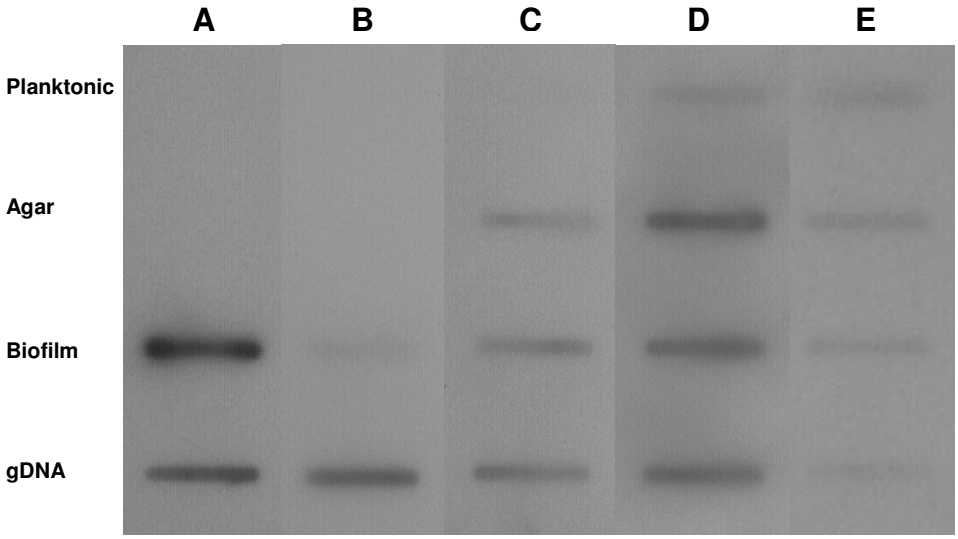

FIG. 4.4. Virtual Northern expression signals of inserts from clones YO12_B4 (A), YO12_B7 (B), YO12_A24 (C), YO12_A31 (D) and YO12_A52 (E) with cDNA (~2 µg) generated from total RNA obtained from planktonic, agar surface-associated and biofilm growth of isolate YO12 (top to bottom), respectively. Genomic DNA (gDNA) was used as positive control.

For isolate YO64, differential expression was observed for inserts from clones YO64_B6, YO64_B23, YO64_B49, YO64_A2 and YO64_A13 (Fig. 4.5, Table 4.3). The quantified x-fold change in density values confirmed differential expression of inserts from clones YO64_B6, YO64_B23, YO64_B49, YO64_A2 and YO64_A13 in each of the biofilm and agar surface-associated phases. No unique gene expression was observed for selected YO64 clone inserts.

Table 4.3. Differential expression patterns of 10 selected YO12 and YO64 clones from biofilm and agar surface-associated phases, respectively, following virtual Northern analysis

Isolate/Growth phase	Clone no.	Protein	Expression			Integrated density fold change					
						In biofilm phase compared to:			In agar surface-associated phase compared to:		
			Plank-tonic	Biofilm	Agar	TUF crl	planktonic	agar	TUF crl	planktonic	biofilm
YO12 Biofilm	YO12_B4	Major facilitator superfamily (MFS) transporter	-	+	-	1.07	199650	199650	-	-	-
	YO12_B7	MATE efflux family protein	-	+	-	-1.42	131404	131404	-	-	-
YO12 Agar	YO12_A24	Putative lipoprotein	+	+	+	-1.09	1.15	1.05	-1.03	1.10	-1.05
	YO12_A31	Hypothetical protein Fjoh_2230	+	+	+	-1.04	1.18	-1.05	1.12	1.24	1.05
	YO12_A52	Putative HlyD family secretion protein	+	+	+	-1.07	-1.01	-1.03	1.07	1.02	1.03
YO64 Biofilm	YO64_B6	GumN Family protein	-	+	+	1.02	277714	1.01	1.03	273986	-1.01
	YO64_B23	Hypothetical protein BACSTE_03846	+	+	+	-1.06	1.35	1.02	-1.05	1.33	-1.02
	YO64_B49	Hypothetical protein BACFIN_00668 Alginate lyase	-	+	+	-1.20	226499	-1.03	-1.14	233415	1.03
YO64 Agar	YO64_A2	Ubiquinone/menaquinone biosynth. methyltransferase	-	+	+	1.10	299808	1.04	1.08	288051	-1.04
	YO64_A13	Diaminopimelate epimerase	+	+	+	-1.07	-1.01	-1.07	1.02	1.06	1.07

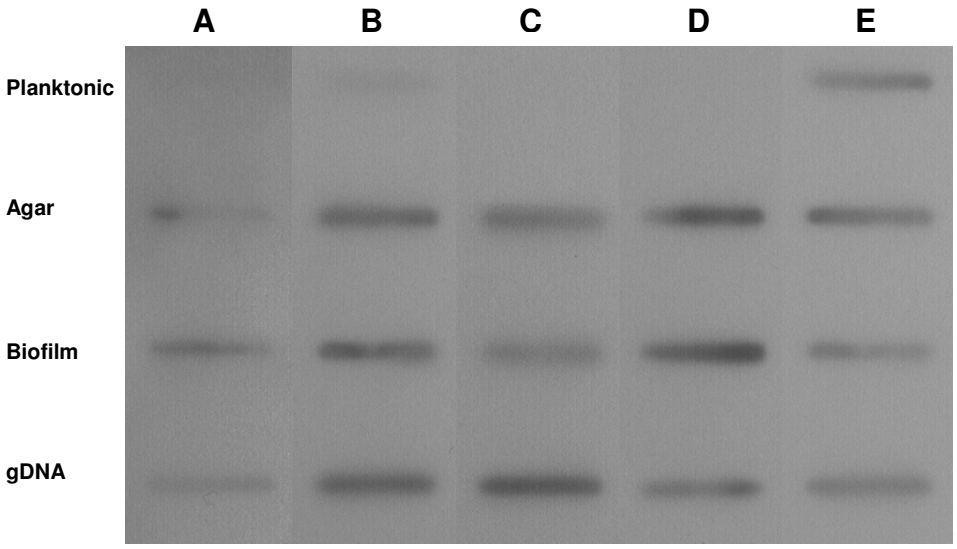

FIG. 4.5. Virtual Northern expression signals of inserts form clones YO64_B6 (A), YO64_B23 (B), YO64_B49 (C), YO64_A2 (D) and YO64_A13 (E) with cDNA (~2 µg) generated from total RNA obtained from planktonic, agar surface-associated and biofilm growth of isolate YO64 (top to bottom), respectively. Genomic DNA (gDNA) was used as positive control.

4.4. DISCUSSION

In the present study, a comparative 2-D/MS proteomic and transcriptomic SSH approach was used to assess the differential gene expression that occur in *F. johnsoniae*-like isolates during biofilm formation. This is the first study to report differential gene expression between the planktonic and sessile phases of *F. johnsoniae*-like isolates. Thirty cloned sequences displaying homology to known proteins were identified using SSH, and were indicative of the physiological changes that occurred in the *F. johnsoniae*-like isolates during the five day biofilm and agar surface-associated incubation period. The significance of the proteins identified in the biofilm and agar surface-associated phases is discussed below.

In *P. putida* biofilm cells, physiological changes in amino acid metabolism, membrane proteins and transporters and proteins involved in EPS production were observed using the SSH technique (Sauer and Camper, 2001). In the present study, similar changes were observed, i.e., differential expression in amino acid metabolism, as well as the identification of membrane-associated proteins involved in transport and secretion of EPS and components of EPS biosynthesis.

Significant differences were observed in the functional properties of cloned insert sequences identified in the biofilm and agar surface-associated phases of isolates YO12 and YO64, respectively. Insert sequences identified in the biofilm phase of isolate YO12 were involved in four different functional categories, i.e., membrane/transport/motility, transcription/translation, adaptation/protection and metabolic processes, while those identified in the biofilm phase of isolate YO64 were involved in membrane/transport/motility, adaptation/protection, metabolic processes and one hypothetical protein (Tables 4.1 and 4.2).

For isolate YO12, 50% of the cloned insert sequences involved in membrane/ transport/motility were those associated with efflux systems, while the remaining two inserts displayed homology to a putative TRAP-like transporter and chloride channel protein (Table 4.1), respectively. The uniquely expressed insert from clone YO12_B4 (Fig. 4.6) displayed 37% homology to the major facilitator superfamily (MFS) transporter, which is an efflux transporter with multiple functions in Gram-negative bacteria. Efflux and secretion systems provide a means of molecular export through the complex membrane of Gram-negative bacteria (Gerlach and Hensel, 2007). These transport systems generally exhibit a broad range of substrate specificity and molecules exported include proteins, toxins, polysaccharides, antimicrobial agents and other toxic compounds (Gerlach and Hensel, 2007; Hinsa *et al*., 2003; Norton *et al*., 2008; Rivas *et al*., 2008; Silver *et al*., 2001; Vila *et al*., 2007). Efflux transporter proteins function in many cellular processes, including osmoregulation, excretion of polysaccharides, motility, biofilm formation, and protection from toxic effects of multiple chemical compounds (Haft *et al*., 2006; Hinsa *et al*., 2003; McBride,

2004; Norton *et al*., 2008; Rivas *et al*., 2008; Silver *et al*., 2001; Vila *et al*., 2007). Up-regulation of transporter proteins may take place during conditions of stress, such as stationary phase growth in the deeper layers of the biofilm, where bacteria are exposed to the accumulation of metabolites and high levels of waste materials that are potentially harmful to cells (Kvist *et al*. 2008). However, up-regulation of efflux pumps is also associated with antibiotic resistance (Kvist *et al*. 2008). One of the major mechanisms of antimicrobial resistance in Gram-negative bacteria involves efflux pumps, mainly of the MFS family (Kvist *et al*. 2008; Vila *et al*., 2007). Pathogenic *Flavobacterium* spp. found in aquaculture settings are well known for their resistance to a wide range of antibiotics (Bernardet and Nagakawa, 2006). Horizontal transfer of these efflux systems have been suggested to take place between Gram-negative bacteria that share the same ecological niche (Ribera *et al*., 2003). Since horizontal transfer of DNA is enhanced within biofilms (Vilain and Brözel, 2006; Whitchurch *et al*., 2002), this may explain the wide distribution of antibiotic resistance, such as tetracycline resistance, in aquatic Gram-negative bacteria (Bernardet *et al*., 2006; Bernardet and Nagakawa, 2006; Kerry *et al*., 1996; Miranda *et al*., 2003).

MFS transporter proteins also play a role in bacterial motility and polysaccharide secretion. In *Myxococcus xanthus*, the multidrug transporter homologue and potential MFS protein MmrA, was demonstrated to play an important role in polysaccharide secretion and A and S motility (Kimura *et al*., 2004). The MFS transporter protein PcaK in *P. putida* functions as a chemoreceptor and is required for aromatic acid chemotaxis (Harwood *et al*., 1994). In addition to the ABC transporter components implicated in *F. johnsoniae* gliding motility (McBride, 2004), MFS transporter proteins may also be involved in the secretion of polysaccharides required for gliding motility as well as for biofilm maturation of *F. johnsoniae*-like isolate YO12.

Insert from clone YO12_B7, which also appeared to be uniquely expressed in the biofilm phase (Fig. 4.6), displayed 55% homology to a multidrug and toxic compound extrusion (MATE) family protein. As with MFS transporters, MATE efflux pumps facilitate resistance to multiple toxic agents in several Gram-

negative bacteria. Members of this efflux family may confer a > 4-fold increase in the minimum inhibitory concentrations (MICs) of norfloxacin, ofloxacin, ciprofloxacin and gentamicin, and a 2-fold increase in the MICs of kanamycin, erythromycin, chloramphenicol, tetraphenylphosphonium chloride and trimethoprim (Su *et al*., 2005). Although these efflux systems may facilitate the export of harmful secondary metabolites and other toxic byproducts in *F. johnsoniae*-like isolate YO12 biofilms, their unique expression and/or significant up-regulation in the biofilm phase may also confer biofilm-associated antimicrobial resistance (Kvist *et al*. 2008).

The clone YO12_B11 insert displayed 27% homology to a putative TRAP-like transporter from *Desulfobacterium autotrophicum*. In *Haemophilus influenzae*, TRAP-like transporters are required for the transport of sialic acid, an important carbohydrate component of lipooligosaccharides in the outer membrane (Johnston *et al*., 2008). Sialic acid is required for resistance to complement-mediated killing and the formation of biofilms *in vitro*, which is important for the survival of *H. influenzae* in the host environment. Sialic acid also forms part of capsular material (Lewis *et al*., 2004), which may be important in adhesion and attachment processes (Crump *et al*., 2001; Norton *et al*., 2008). Hence, the putative TRAP-like transporter might play a role in the transport of important carbohydrate components of outer membrane and/or capsular material in *F. johnsoniae*-like isolate YO12 and may play a role in adhesion/attachment and/or aggregation of cells (Donlan, 2002; Hall-Stoodley and Stoodley, 2002; Stanley and Lazazzera, 2004; Wang *et al*., 2004b; Yildiz and Schoolnik, 1999).

The insert from clone YO12_B58 displayed 81% homology to the hypothetical protein Fjoh_0539 and 33% homology to a chloride channel protein, an anion channel with diverse cellular functions, which range from osmoregulation to signal transduction, transport and intracellular pH regulation (Jentsch and Günther, 1996), respectively. Chloride channels import glutamate to counter the proton flow when stationary phase cells encounter conditions of acid shock (Iyer *et al*., 2002). Glutamate is decarboxylated to form GABA with the consumption of protons in a reaction involving glutamate synthase (clone

YO12_B30 insert). These components may be required for osmoregulation in the deeper or base layers of biofilm growth, where isolate YO12 cells are likely to encounter osmotic stress.

Insert sequences involved in adaptation/protection included putative proteins involved in osmoregulation and calcium-binding, as well as a bacteriophage T4 analogue and a putative peptide synthetase (Table 4.1). Clone YO12_B30 insert displayed 95% homology to the NADH/NADPH subunit of glutamate synthase, an essential enzyme in the glutamate synthesis pathway. Glutamate may accumulate in osmotically stressed cells, such as the stationary phase or growth-arrested cells in the deeper layers of the biofilm, which forms part of the general stress response and plays a role in osmoregulation (Saum and Müller, 2007). However, glutamate biosynthesis may also have other, less clear functional properties in biofilm growth. Pysz *et al*. (2004) demonstrated the up-regulation of the three glutamate synthase components responsible for the formation of an iron-sulfur cluster binding complex, as well as the NADH oxidase subunit in the *Thermotoga maritima* biofilm phase. Interestingly, O'Toole and Kolter (1998b) demonstrated the restoration of attachment of *Pseudomonas fluorescens* biofilm-defective mutants on glutamate- and iron-enriched media. Although glutamate biosynthesis may form part of osmoregulation in *F. johnsoniae*-like isolate YO12 biofilm cells, it may also induce biofilm formation as observed for *P. fluorescens* biofilms (O'Toole and Kolter, 1998b).

Clone YO12_B46 insert displayed 47% homology to the hypothetical protein BACCOPRO_01435 and 25% homology to a GPW/gp25 family protein, a putative lysozyme of bacteriophage T4, respectively. Bacterial protein analogues to bacteriophage T4 tail and base plate proteins have been demonstrated in bacteria that harbour type IV secretion systems (Boyer *et al*., 2009). Components of type IV secretion systems associated with conjugative transposons were identified by *F. johnsoniae* UW101 genome sequence analysis (McBride *et al*., 2009). These proteins are speculated to play a role in DNA and protein translocation. Possible up-regulation of a putative GPW/gp25 family protein in the biofilm phase of isolate YO12, may play a role in processing and/or

may be involved in the translocation of proteins required for attachment and biofilm formation.

The clone YO12_B52 insert displayed 33% homology to an EF hand domain-containing protein, associated with Ca^{2+} binding, Ca^{2+} transport and Ca^{2+} signal transduction in a number of Gram-positive and Gram-negative bacteria (He *et al*., 2008; Michiels *et al*., 2002). Calcium-binding proteins are involved in a variety of cellular functions, including initial attachment and biofilm formation in several pathogens, including *Staphylococcus aureus*, *Enterococcus faecalis*, *S. mutans*, *P. aeruginosa* and *Salmonella* spp. (Gerlach and Hensel, 2007; He *et al*., 2008; Lasa, 2006). The BAP family proteins, such as Bap in *S. aureus*, contain several EF hand motifs and are well known for their function in attachment and biofilm formation (Cucarella *et al*., 2001; Gerlach and Hensel, 2007; He *et al*., 2008; Lasa, 2006). Similarly, the EF hand domain protein CiaX, sharing high amino acid sequence similarity to Bap, is required for calcium-mediated biofilm formation in *S. mutans* (He *et al*., 2008). Thus, the clone YO12_B52 insert may encode a protein involved in Ca^{2+} signal transduction and biofilm formation in *F. johnsoniae*-like isolate YO12.

The clone YO12_B59 insert displayed 31% homology to a peptide synthetase, an enzyme present in Gram-negative and Gram-positive bacteria involved in the production of antimicrobial agents and products with surface-conditioning properties required for swarming motility and biofilm formation (Eberl *et al*., 1999; Lewandowski and Beyenal, 2007; Neu, 1996). In *Serratia liquefaciens*, a gene encoding a putative peptide synthetase was responsible for the production of a conditioning film required for swarming motility (Eberl *et al*., 1999). Similarly, biofilm formation of isolate YO12 on abiotic surfaces may be dependent on the production of conditioning films by peptide synthetases.

Insert sequences involved in transcription/translation included a putative transcriptional regulator and a putative ribonuclease H (Table 4.1). Clone YO12_B2 insert displayed 97% homology to a protein of the AsnC family of transcriptional regulators. The Lrp/Asn family of transcriptional regulators influences DNA structure (Beloin *et al*., 2003; Thaw *et al*., 2006) and cellular

metabolism, often through binding amino acid effectors regulating gene expression (Kroos, 2007; Thaw *et al*., 2006). Lrp/Asn family proteins and similar transcriptional factors may influence complex regulatory systems, such as fruiting body formation in *M. xanthus* and sporulation in *Bacillus subtilis* (Kroos, 2007). Up-regulation of a putative AsnC family transcriptional regulator may play a role in regulated gene expression in the biofilm mode of *F. johnsoniae*-like isolate YO12.

Clone YO12_B9 insert displayed 91% homology to ribonuclease H, an enzyme that cleaves the RNA strand from RNA-DNA hybrids. These enzymes may also function in DNA repair and transcription (Malik and Eickbush, 2009). Possible up-regulation of a putative ribonuclease H in the biofilm phase of isolate YO12 may be directly related to the increased transcriptome associated with biofilm growth (Ghigo, 2003; Sauer *et al*., 2002).

Insert sequences involved in metabolic processes included a putative chitinase and an AMP-binding enzyme (Table 4.1). Clone YO12_B52 insert also displayed 45% homology to a putative chitinase, an enzyme involved in chitin degradation as a carbon source. *F. johnsoniae* is well known for its ability to degrade chitin (Bernardet and Nagakawa, 2006, McBride, 2004; McBride *et al*., 2009). Chitin degradation is positively correlated with gliding motility in this organism (McBride, 2004; Chapter 2). However, gliding motility and chitinolytic ability are negatively correlated with biofilm formation in *F. psychrophilum* (Álvarez *et al*., 2006) and *F. johnsoniae*-like isolates (Basson *et al*., 2008; Chapter 2). While the chitinolytic ability of *F. johnsoniae*-like isolates is repressed in initial attachment/early stage biofilm cells, the up-regulation of a putative chitinase in the mature biofilm might reflect the nutrient acquisition mechanism of *F. johnsoniae*-like biofilm cells.

Clone YO12_B59 insert also displayed 35% homology to an AMP-binding enzyme, an enzyme likely to be involved in energy metabolism. AMP-binding enzymes play an important role in energy homeostasis of cells. These enzymes are up-regulated in eukaryotic cells during stress conditions that deplete ATP

(Cheung *et al*., 2000). Nutrient limitations in the base layer of YO12 biofilm cells may induce AMP-binding proteins to compensate for ATP depletion.

While no putative efflux systems were identified in the biofilm phase of isolate YO64, cloned insert sequences involved in membrane/transport/motility displayed homology to putative adhesins and/or motility proteins (Table 4.2). Clone YO64_B60 insert displayed 39% homology to a putative RagB/SusD domain protein, 31% homology to a SusD homologue from *Bacteroides thetaiotaomicron* and 27% homology to a putative lipoprotein, respectively. In *B. thetaiotaomicron*, the lipoprotein SusD and OMP SusC constitute outer membrane polysaccharide-binding proteins that may be important in cellular attachment to mucus glycans (Pumbwe *et al*., 2006). *F. johnsoniae* genome sequence analysis revealed 42 *susD*-like genes involved in polysaccharide binding and/or degradation (McBride *et al*., 2009). Glycoproteins, involved in polysaccharide binding, have been demonstrated to play a role in cellular attachment of *F. columnare* (Decostere *et al*., 1999a) and *F. psychrophilum* (Møller *et al*., 2005), respectively, and lipoproteins have also been associated with gliding motility in *F. johnsoniae* (McBride, 2004). Moreover, the *susD*-like genes encode proteins components of two-component regulatory systems which may influence biofilm processes (Ghigo, 2003). Hence, the putative SusD-like protein encoded by the gene sequence in clone YO64_B60 may well play a role in the adhesion or attachment process of isolate YO64 to diverse surfaces.

Clone YO64_B69 insert displayed 85% homology to a putative SprD protein of *F. johnsoniae* and 45% homology to the SprD precursor, a putative cell surface protein of *F. psychrophilum*, respectively. Two other *spr* gene products, SprA and SprB, a cell-surface protein and adhesin, respectively, have been demonstrated to be involved in the spreading phenotype and gliding motility of *F. johnsoniae* (McBride *et al*., 2009; Nelson *et al*., 2008; Nelson *et al*., 2007). The possible up-regulation of a putative SprD protein suggests that the ability to spread on a surface, rather than gliding motility, would be critical for the initial attachment stages of biofilm formation. OMPs and cell surface proteins, such as SprB, involved in motility may also play a role in adhesion/attachment and signal

transduction (Gerlach and Hensel, 2007; McBride, 2004; McBride *et al*., 2009; Van Houdt and Michiels, 2005). The membrane-bound SprD protein might function in adhesion/attachment processes involved in motility, spreading on surfaces (biotic and abiotic) and may thus also be functional in surface colonization and biofilm formation of isolate YO64.

Of the cloned insert sequences involved in adaptation/protection, 67% were putative polysaccharide biosynthesis proteins, while one insert encoded a putative glutamyl-tRNA reductase (Table 4.2). Clone YO64_B6, which appeared to be up-regulated in the biofilm and agar surface-associated phases (Fig. 4.7), displayed 43% homology to a GumN family protein, a protein with unknown function. In the genus *Xanthomonas*, twelve gum genes are associated with the biosynthesis and secretion of the xanthan gum polysaccharide (Pollock *et al*., 1997). Xanthan gum forms part of the EPS produced by *Xanthomonas* spp., which plays an important role in virulence and biofilm formation (Lu *et al*., 2008; Pollock *et al*., 1997). Although *gumN* is co-transcribed with the *gumB-M* operon, the GumN protein displays no similarity to known proteins and appears not to be essential for xanthan gum production or excretion in *Xanthomonas campestris* (Lu *et al*., 2008; Pollock *et al*., 1997). Although gum family genes have not yet been identified in *F. johnsoniae*, genome sequence analysis has identified many polysaccharide biosynthesis genes, including many hypothetical proteins predicted to be involved in exopolysaccharide production/export (McBride *et al*., 2009). The up-regulation of a putative GumN family protein in biofilm and agar surface-associated cells of isolate YO64 may be involved in EPS production/secretion required for surface colonization of abiotic and biotic surfaces. This also highlights the fact that while EPS elucidation is repressed during the early stages of biofilm formation, EPS elaboration is necessary for biofilm maturation.

Insert from clone YO64_B49, which was also up-regulated in the biofilm and agar surface-associated phases (Fig. 4.7), displayed 37% homology to the hypothetical protein BACFIN_00668 and 31% homology to an alginate lyase, an enzyme that degrades the extracellular polysaccharide alginate. Alginate

production in *P. aeruginosa* is associated with mucoid strains, virulence and a large change in gene expression in the biofilm state (Boyd and Chakrabarty, 1994; Sutherland, 2001). The production of alginate lyase is accompanied by cell detachment and mainly associated with the dispersal stage of *P. aeruginosa* biofilms (Boyd and Chakrabarty, 1995). Alginate biosynthesis and degradation enables the development, maintenance and spread of *P. aeruginosa* biofilms (Boyd and Chakrabarty, 1995). In *F. psychrophilum*, four proteins similar to the alginate O-acetyltransferases of *P. aeruginosa* potentially play a role in biofilm formation (Duchaud *et al*., 2007). The production of a putative alginate lyase enzyme in the biofilm and agar surface-associated phases of the *F. johnsoniae*-like isolate YO64 (Fig. 4.4) may be indicative of dispersal and/or slough mechanisms of sessile cells.

Clone YO64_B35 insert displayed 62% homology to glutamyl-tRNA reductase, an enzyme that reduces glutamyl-tRNA to glutamate-1-semialdehyde (Moser *et al*. 2001). Although aminoacyl-tRNA synthetases catalyze the tRNA-mediated activation of amino acids, this reaction also initiates tetrapyrrole biosynthesis, required for aerobic respiration, in many prokaryotes (Moser *et al*. 2001). The presence of actively dividing cells in the surface-layers of YO64 biofilm growth may explain the up-regulation of respiratory components. However, oxygen/nutrient gradients in multi-layered biofilm growth may also lead to oxygen-poor areas or osmotic stress in the deeper layers (Becker *et a*l., 2001), which may induce the production of respiratory components for increased oxygen binding or glutamate as part of osmoregulation (Saum and Müller, 2007).

Clone YO64_B68 insert displayed 83% homology to NAD(P)H quinone dehydrogenase, an enzyme involved in oxidative stress. Quinones play a vital function in respiration, oxidative stress management and gene regulation (Soballe and Poole, 1999). In *Streptococcus gordonii*, NAD(P)H quinones are essential for healthy biofilms and form part of a putative oxidative stress response operon (Loo *et al*., 2004). Similarly, possible up-regulation of a putative NAD(P)H quinone dehydrogenase in isolate YO64 biofilm cells may be

required for healthy cell growth and/or oxidative stress management in the stationary phase layer of cells.

Cloned insert sequences involved in metabolic processes included a putative glycoside hydrolase, as well as a protein involved in amino acid metabolism (Table 4.2). Clone YO64_B4 insert displayed 82% homology to a glycoside hydrolase family protein, which are enzymes involved in polysaccharide degradation. Glycoside hydrolases often play a role in the dispersal of cells from the mature biofilm (Ramasubbu *et al*., 2005). Released cells can attach to surrounding areas or other surfaces enabling the biofilm to spread. In *Aggregatibacter actinomycetemcomitans* biofilms, the glycoside hydrolase DspB is required for *N*-acetylglucosamine-containing extracellular polysaccharide degradation leading to cell dispersal as well as the disaggregation of large cell clumps (Ramasubbu *et al*., 2005). In *S*. *gordonii*, beta-glucosides were induced in the biofilm phase and it was proposed that besides the metabolic function of these enzymes, they may also be involved in the synthesis of cell surface glycoproteins and polysaccharides required for attachment and biofilm formation (Kiliç *et al*., 2004). Hence, besides its function in cell dispersal, the putative glycoside hydrolase may be involved in the biosynthesis of glycoproteins, such as SprB, which are functional as adhesins in *F*. *johnsoniae* (McBride *et al*., 2009) and *F*. *johnsoniae*-like isolate YO64.

Clone YO64_B5 insert displayed 96% homology to DAHP synthetase I, an enzyme that functions in amino acid metabolism. DAHP synthetases catalyze the first step in the biosynthesis of aromatic acids, including amino acids, and vitamins (Ger *et al*., 1994). Becker *et al*. (2001) observed up-regulation of aminoacyl-tRNA synthetase in *S*. *aureus* biofilm cells, which was subsequently down-regulated with threonine supplementation. Hence, nutrient limitation of YO64 biofilm cells existing in a starved state may lead to the induction of enzymes involved in amino acid metabolism as an alternative carbon source (Seidl *et al*., 2009).

The putative insert gene products differentially expressed and/or uniquely expressed in the biofilm phase of the *F*. *johnsoniae*-like isolates YO12 and YO64

were classified under five major categories, viz., hypothetical, membrane proteins, metabolic processes, adaptation/protection and transcription/translation. Similar to the results obtained by 2-D/MS analysis (Chapter 3), the functional categories concurred in general with three major classes previously described for differentially expressed genes in the biofilm phase, viz., membrane proteins, metabolic processes and adaptation/protection (Jouenne *et al*., 2004), as well as a fourth class, i.e., transcription/translation. Shemesh *et al*. (2007) observed a substantial number of up-regulated genes involved in transcription in the biofilm phase of *S*. *mutans*. The up-regulation of transcriptional factors and other putative proteins categorized under transcription/translation may form part of the adaptive response (Helloin *et al*., 2003) and also indicates regulated gene expression as part of the biofilm phenotype.

Membrane proteins, including putative transporters, putative lipoproteins and other cell surface components, with diverse functional properties appeared to be up-regulated in the biofilm phase of both isolates YO12 and YO64. These cell surface structures are likely to be involved in attachment, signal transduction, EPS biosynthesis/secretion and/or motility (Haft *et al*., 2006; McBride, 2004; Norton *et al*., 2008; Silver *et al*., 2001).

Four proteins likely to be involved in polysaccharide biosynthesis/secretion were shown to be up-regulated or uniquely expressed in biofilm cells of *F*. *johnsoniae*-like isolates YO12 and YO64 (Figs. 4.3 and 4.4). Extracellular polysaccharides and EPS production plays an integral role in the processes of irreversible attachment, microcolony formation and biofilm development (Donlan, 2002; Hall-Stoodley and Stoodley, 2002; Otto *et al*., 2001; Seyer *et al*., 2005; Stanley and Lazazzera, 2004; Tsuneda *et al*., 2003; Van Houdt and Michiels, 2005; Wang *et al*., 2004b). In *E*. *coli*, the *pga*-dependent cell-bound polysaccharide was shown to affect biofilm development by promoting abiotic surface attachment and intercellular adhesion (Wang *et al*., 2004b). The *Vibrio* polysaccharide, VPS, produced by the rugose variant of *V*. *cholerae* enhances the biofilm-forming capacity of this phenotype (Beyhan *et al*., 2007). In *B*. *subtilis*, the *epsA-O* operon, consisting of 15 genes encoding the biosynthesis of

exopolysaccharide, is required for complex biofilm growth (Chu *et al*., 2006). Besides affecting colony spreading in *F. johnsoniae* (Godchaux *et al*., 1991), polysaccharides also play a role in the gliding motility of *M. xanthus* and cyanobacteria (McBride, 2004; Yu and Kaiser, 2007). In the present study, a statistically significant positive correlation was observed between ECC and gliding motility of *F. johnsoniae*-like isolates (Chapter 2), however, a statistically significant negative correlation was observed between ECC and abiotic surface adherence.

The microtiter adherence assay was used to investigate 24 h biofilm growth of *F. johnsoniae*-like isolates (Basson *et al*., 2008), while differential gene expression using SSH was performed on 5 day old biofilm and agar surface-associated cultures. Hence, differential gene expression of the 5 day old biofilm, representing a mature biofilm with more elaborated EPS, would differ considerably from that of a 24 h (much younger) biofilm. Sauer *et al*. (2002) also demonstrated that the most profound differences in protein expression occurred in the maturation-2 phase (6 day old biofilm) of *P. aeruginosa* biofilm cells, when compared to planktonic and maturation-1 (1 day old biofilm) biofilm cells. While polysaccharide production may not be an essential component of the young biofilm, it may be necessary for the mature biofilm structure, which would lead to up-regulation of polysaccharide biosynthesis genes. The presence of putative polysaccharide lyases (clones YO64_B4 and YO64_B49) might also suggest that cell dispersal from the mature biofilm was underway.

Three insert genes from clones YO12_B30, YO12_B58 and YO64_B35, displayed homology to proteins associated with intracellular glutamate regulation. Although glutamate metabolism is closely associated with osmoregulation during conditions of osmotic stress (Saum and Müller, 2007), it may also induce biofilm formation as part of a survival strategy in soil bacteria (O'Toole and Kolter, 1998b). O'Toole and Kolter (1998b) demonstrated that glutamate, along with iron and citrate, respectively, restored biofilm formation of surface attachment defective *P. fluorescens* cells and suggested that plants may promote the formation of biofilms on their roots by excretion of glutamate. Up-regulation of

five genes involved in glutamate synthesis and expression of glutamate synthetase components involved in iron binding was also observed in biofilm growth of the geothermophilic bacterium *T. maritime* (Pysz *et al*., 2004). In *B. subtilis*, polyglutamate is a major extracellular polymeric substance responsible for biofilm formation on the liquid/air interface (Morikawa *et al*., 2006). The role of glutamate as a biofilm signal molecule is speculative and further investigation is needed to clarify the reason for possible up-regulation of putative proteins involved in glutamate metabolism of the *F. johnsoniae*-like isolates.

While no quorum sensing system has been identified in *F. johnsoniae* and other *Flavobacterium* spp., it is not unlikely that environmental chemicals/stimuli, such as glutamate, might act as triggers that stimulate biofilm growth. Other signal transduction and gene regulatory systems, involving sigma factor proteins, have been identified in *F. johnsoniae* (McBride *et al*., 2009). However, none of these proteins were identified in this study. Only a single putative SusD homologue, which forms part of the *susD*-like genes involved in the production of regulatory proteins, including members of the two-component regulatory systems LuxR/winged helix 431 regulators, LacI-type repressors, XRE-like repressors, and AraC family regulators (McBride *et al*., 2009), was identified in the biofilm phase of isolate YO64.

Although proteomic investigations of agar surface-associated cells have demonstrated alteration in the proteome compared to planktonic and/or biofilm cells (Mikkelsen *et al*., 2007; Chapter 3), the transcriptome of this phase has not been extensively studied with advanced transcriptomic techniques, such as SSH. In *B. subtilis*, the major gene regulatory system SinR was identified with mutational genetic analysis of agar surface-associated cells (Hamon and Lazazzera, 2001). Wang *et al*. (2004a) demonstrated that agar surface-associated cells of *Salmonella typhimurium* display a markedly different physiology from that of planktonically grown cells by using microarray analysis. Resch *et al*. (2005) also demonstrated differential gene expression between planktonically grown and agar surface-associated *S. aureus* cells using microarray analysis. Proteins involved in membrane and polysaccharide

biosynthesis and export were significantly up-regulated in agar surface-associated *S. aureus* cells (Resch *et al*., 2005). In the present study, differential expression, with possible up-regulation of several membrane components and proteins involved in polysaccharide biosynthesis/secretion were demonstrated in the agar surface-associated growth phase of the *F. johnsoniae*-like isolates YO12 and YO64. To our knowledge, this is the first study to report gene expression of agar surface-associated cells using the SSH transcriptomic approach.

Cloned insert sequences identified in the agar surface-associated phase of isolate YO12 were involved in two different functional categories, i.e., membrane/transport/motility and metabolic processes, while those identified in the agar surface-associated phase of isolate YO64 were involved in membrane/transport/motility, metabolic processes and adaptation/protection (Tables 4.1 and 4.2). For isolate YO12, 75% of membrane proteins/ transport/motility inserts were putative efflux transporters, while one insert displayed homology to a putative lipoprotein (Table 4.1).

Clone YO12_A24 insert displayed 29% homology to a putative lipoprotein with unknown function, which appeared to be up-regulated in the biofilm and agar surface-associated phases (Fig. 4.6). The *F. johnsoniae* genome is predicted to encode 423 lipoproteins (McBride *et al*., 2009). Lipoproteins, such as SusD and the five Gld lipoproteins, are generally directed to the inner and outer membrane where they are involved in complex cellular processes such as protein folding, virulence, signal-transduction, motility, polysaccharide binding/secretion, cell adhesion and biofilm formation (Babu *et al*., 2006; D'Andrea and Regan, 2003; Koo *et al*., 2008; Van Schaik *et al*., 2005; Yu and Kaiser, 2007). The putative lipoprotein up-regulated in the agar surface-associated phase of isolate YO12 is likely to be involved in adhesion and/or polysaccharide binding/secretion required for surface colonization.

Clone YO12_A31 insert displayed 54% homology to the hypothetical protein Fjoh_2230 and 27% homology to an efflux ABC transporter permease protein. Although this protein was expressed in all three growth phases,

expression was up-regulated in the biofilm and agar surface-associated phases, with highest expression in the latter (Fig. 4.6 and Table 4.3). ABC transporters have been linked to motility in *M. xanthus* and *F. johnsoniae* (Kimura *et al*., 2004; Nelson and McBride, 2006). ABC transporters have also been demonstrated to play an important role in bacterial attachment and export of EPS required for biofilm formation (Hinsa *et al*., 2003; Silver *et al*., 2001). The putative ABC transporter is likely to play a role in motility (McBride, 2004), bacterial attachment, the excretion of polysaccharide and EPS required for biofilm formation (Haft *et al*., 2006; Norton *et al*., 2008; Silver *et al*., 2001), as well as antimicrobial resistance (Vila *et al*., 2007) in this *F. johnsoniae*-like isolate.

Clone YO12_A32 insert displayed 90% homology to a resistance-nodulation-division (RND) family efflux transporter MFP subunit, which forms part of the RND/MFP/OMF efflux system. Similarly, the insert from clone YO12_A52, up-regulated in the biofilm and agar surface-associated phases, with highest expression in the latter (Fig. 4.6 and Table 4.3), displayed 60% homology to a putative HlyD family secretion protein and 53% homology to a RND family efflux transporter, MFP subunit. The HlyD protein is a membrane-bound component, the RND component in RND/MFP/OMF efflux systems, or the MFP component in ABC/MFP/OMP secretion systems (Gerlach and Hensel, 2007; Poole, 2001). In RND efflux systems, the HlyD protein forms a fused domain similar to the PilZ protein in Alg44 family proteins, which functions in c-di-GMP regulation (Amikam and Galperin, 2006). Amikam and Galperin (2006) proposed that the association of the second messenger c-di-GMP with efflux systems may explain its role in protein secretion and export of EPS required in biofilm formation. However, *F. johnsoniae* does not appear to use the second messenger c-di-GMP (McBride *et al*. 2009).

RND family proteins have been identified in all major kingdoms, but seem to confer multidrug resistance only in Gram-negative microorganisms (Poole, 2001). The association of antimicrobial resistance with the biofilm lifestyle may in part be a result of the up-regulation of diverse efflux systems (Kvist *et al*., 2008). As discussed above for the efflux systems identified in the biofilm phase of

isolate YO12, the putative ABC transporter and RND efflux components in the agar surface-associated phase may be involved in the removal of secondary metabolites and waste products in the stationary phase layer of cells, which may also lead to increased antimicrobial resistance.

Insert sequences involved in metabolic processes included a putative polysaccharide biosynthesis protein, as well as a protein involved in amino acid metabolism (Table 4.1). Clone YO12_A4 insert displayed 38% homology to a polysaccharide biosynthesis protein from *Methanosphaera stadtmanae*. Polysaccharides are important components of biofilms and contribute directly to biofilm properties (Sutherland, 2001). Exopolysaccharide composition and production may vary greatly between different bacterial species. Alginate and colonic acid seem to be the main polysaccharide component associated with *P. aeruginosa* and *E. coli* biofilms, respectively (Boyd and Chakrabarty, 1994; Sutherland, 2001). The *Vibrio* polysaccharide, VPS, produced by the rugose variant of *V. cholerae* enhances the biofilm-forming capacity of this phenotype (Beyhan *et al*., 2007). Surface-associated growth and colony spreading of bacteria and *F. johnsoniae* in particular, is associated with increased EPS production (Beyhan *et al*., 2007; Godchaux *et al*., 1991; Kives *et al*., 2006). However, the polysaccharide composition of flavobacterial biofilms has not been defined as yet.

Clone YO12_A60 insert displayed 97% homology to a delta-1-pyrroline-5-carboxylate dehydrogenase, an enzyme that functions in amino acid metabolism. Bacteria contain several genes that code for amino acid dehydrogenases, enzymes responsible for amino acid degradation, which may function in carbon metabolism as a secondary carbon source in some organisms (Seidl *et al*., 2009). The possible up-regulation of putative proteins involved in amino acid metabolism may indicate nutrient limitation in the mid- and/or surface-layer of agar surface-associated YO12 cells, forcing the cells to metabolize amino acids as alternative carbon source.

Similar to the biofilm phase of isolate YO64, cloned insert sequences involved in membrane/transport/motility in the agar surface-associated phase

displayed homology to putative adhesins and/or motility proteins (Table 4.2). The clone YO64_A36 insert displayed 36% homology to a cell-surface large adhesion. Adhesins are involved in adherence of microorganisms to biotic and abiotic surfaces, may promote survival and form an integral part of virulence in bacterial pathogens (Gerlach and Hensel, 2007). Cell-surface adhesins recognize a variety of substrates, including living tissue, and play an important role in autoaggregation, bacterial adherence and biofilm formation (Gerlach and Hensel, 2007). Non-fimbrial adhesins play an important role in bacterial attachment and include OMPs, porins, transporter proteins and cell surface proteins, such as the BAP family proteins (Gerlach and Hensel, 2007). The cell-surface large adhesin-like protein identified in the agar surface-associated phase of isolate YO64 may represent a non-fimbrial adhesin which facilitates autoaggregation and adhesion of isolate YO64 cells to the agar surface. Basson *et al*. (2008) observed that autoaggregation of *F. johnsoniae*-like isolates could not be correlated with a specific biofilm phenotype. Nevertheless, adhesins, such as the putative cell-surface large adhesin identified here, may still be involved in aggregation of genetically identical *F. johnsoniae*-like isolates. The importance of this protein and other cell-surface adhesins in *F. johnsoniae* biofilm formation is unknown. Understanding adhesin expression and function in bacteria is incomplete and future research is required to examine the role of adhesins in pathogenic organisms, including *Flavobacterium* spp.

Clone YO64_A41 insert displayed 82% homology to a putative lipoprotein of *F. johnsoniae* with unknown function. Similar to the clone YO12_A24 insert, the putative lipoprotein in the agar surface-associated phase of isolate YO64 may play an important role in adhesion and/or polysaccharide binding/secretion for surface colonization of this isolate.

Insert sequences involved in metabolic processes included a putative diaminopimelate epimerase involved in amino acid metabolism, as well as a putative transketolase involved in nucleotide biosynthesis (Table 4.2). The clone YO64_A13 insert, which appeared to be up-regulated in the agar surface-associated phase (Fig. 4.7), displayed 84% homology to a diaminopimelate

epimerase, an amino acid racemase which catalyzes the interconversion of L,L and D,L-*meso*- diaminopimelate, the precursor to L-Lysine (Koo *et al*., 2000). In *Bordetella pertussis*, a nucleoside epimerase/dehydrogenase, possibly involved in cell envelope biosynthesis, was demonstrated to be expressed only in the biofilm phase (Serra *et al*., 2008). Interestingly, expression patterns of clone YO64_A13 insert displayed expression in all three growth phases (Fig. 4.5), with possible higher expression levels in the agar surface-associated phase (Table 4.3). The reason for possible up-regulation of a putative diaminopimelate epimerase in the agar surface-associated phase of isolate YO64 is not clear.

Clone YO64_A58 insert displayed 98% homology to a transketolase, an enzyme functioning in the pentose phosphate pathway. Transketolase feeds glycolytic intermediates into the pentose phosphate pathway for the production of nucleotides (Rathsam *et al*., 2005a). The induced expression of transketolases has been demonstrated during conditions of stress, such as cold shock in *E. coli* (White-Ziegler *et al*., 2008), and biofilm formation of *S. mutans* (Rathsam *et al*., 2005a). Similarly, the possible up-regulation of a putative transketolase may be the result of stress response in the stationary phase or growth arrested cell layer of agar surface-associated YO64 cells.

The YO64 cloned insert sequences identified in the agar surface-associated phase were predominantly (43%) associated with adaptation/protection and included proteins involved in stress response, as well as a putative methyltransferase involved in diverse cellular processes (Table 4.1). Clone YO64_A2 insert, which was up-regulated in the biofilm and agar surface-associated phases, displayed 75% homology to a ubiquinone/menaquinone methyltransferase, an enzyme that functions in the biosynthesis of ubiquinone and/or menaquinone, components of the respiratory electron transfer chain. Ubiquinone is a bacterial respiratory quinone, an essential component of aerobic respiration, with antioxidant activity (Loo *et al*., 2004). In *E. coli*, ubiquinone/menaquinone deprivation led to accumulation of DsbA and DsbB, components of a disulfide bond formation system, in their reduced forms (Kobayashi *et al*., 1997). Interestingly, *E. coli* DsbA mutant cells

also displayed impaired biofilm formation (Lee *et al*. 2008). A putative thiol, disulfide interchange protein was also identified in biofilm cells of isolate YO12 using 2-D gel electrophoresis in Chapter 3. Up-regulation of quinones in the agar surface-associated and biofilm phases of isolate YO64 (Fig. 4.4) may indicate an oxidative stress response and may function in the maintenance of catalytic proteins in their oxidized state, such as in the case of DsbA and DsbB, which are required for biofilm formation (Lee *et al*. 2008; Loo *et al*., 2004).

Clone YO64_A24 insert displayed 87% homology to the competence/damage-inducible protein CinA, a protein usually associated and/or co-expressed with *recA* (Narumi *et al*., 1999). The expression of these genes is enhanced during competence and the gene products function in recombination (Narumi *et al*., 1999). Dagkessamanskaia *et al*. (2004) proposed that competence in *Streptococcus pneumoniae* may increase virulence and/or may lead to autolysis of stationary phase cells. The reason for possible up-regulation of this protein in the agar surface-associated phase of isolate YO64 may be part of a general stress response associated with stationary phase cells in the population.

Clone YO64_A44 insert displayed 80% homology to a putative SAM-dependent methyltransferase, an enzyme that functions in the methylation of nucleic acids, lipids and proteins (Martin and McMillan, 2002). Methylation of biologically active molecules may influence cellular processes such as biosynthesis, metabolism, signal transduction, protein sorting and repair and nucleic acid processing (Martin and McMillan, 2002). In *Vibrio vulnificus*, inactivation of a *N*-methyltransferase resulted in a loss of type IV pili and the ability to secrete certain enzymes, including metalloproteases and chitinases, and significantly reduced virulence and biofilm formation (Paranjpye and Strom, 2005). However, up-regulation of methyltransferase expression was also demonstrated to form part of the general stress response in *E. coli* (White-Ziegler *et al*., 2008). The diverse functional properties of this enzyme might be required for surface colonization of isolate YO64. However, possible up-regulation of this

protein may also be part of the stress response associated with stationary phase cells.

The putative gene products differentially expressed in the agar surface-associated phase of the *F. johnsoniae*-like isolates YO12 and YO64 were classified under three major categories, viz., membrane proteins, metabolic processes and adaptation/protection. Interestingly, none of the putative proteins identified in the agar surface-associated phase represented transcriptional factors/regulators, as observed for the biofilm phase.

A large number of cell surface/membrane proteins were observed in the agar surface-associated cells of isolates YO12 and YO64, respectively. The abundance of membrane proteins in the biofilm and agar surface-associated phases suggests that large alterations in cell envelope physiology occur in *F. johnsoniae*-like cells on surface contact. This is in accordance with the findings of Pitta *et al*. (1993) who demonstrated that peptidoglycan sacculi of surface-associated *F. johnsoniae* cells were associated with a large amount of protein, whereas planktonic cells had very few or no attached protein. Sauer and Camper (2001) also demonstrated the up-regulation of several membrane-associated proteins in *P. putida* following attachment. Membrane proteins play an important role during the attachment process and early biofilm development (Sauer and Camper, 2001). Among the membrane-associated proteins up-regulated in the biofilm phase of *P. putida*, several displayed homology to efflux pump components, including ABC transporter proteins, and proteins involved in polysaccharide biosynthesis. In the present study, SSH revealed up-regulation and/or unique expression of several putative efflux transporters, including an ABC transporter, and polysaccharide biosynthesis/secretion components in the agar surface-associated as well as biofilm growth phases of *F. johnsoniae*-like isolates. Moreover, Sauer and Camper (2001) identified genes with similarities to virulence factors, antibiotic resistance and adhesins functional in the colonization of biotic and abiotic surfaces. Whiteley *et al*. (2001) also observed the up-regulation of membrane-associated antibiotic resistance factors in biofilm growth of *P. aeruginosa* and suggested that biofilm mode induces resistance to

antimicrobial agents. In the present study, putative gene products that are likely to play a role in virulence, antimicrobial resistance and attachment were also identified.

Stress conditions may result in overexpression of efflux pumps, which are often associated with bacterial multidrug resistance (Vila *et al*., 2007). Six families of efflux-pump-mediated resistance systems exist, viz., ABC family, the MFS, the RND family, the MATE family, the SMR family and the drug/metabolite transporter (DMT) superfamily (Vila *et al*., 2007). Inserts of five isolate YO12 clones displayed homology to efflux proteins belonging to the MFS, MATE, ABC and RND families, respectively (Table 4.1). In the previous chapter, 2-D analysis also revealed a putative ABC transporter protein in the biofilm phase of isolate YO12. Efflux transporters appear to be important components of biofilm and agar surface-associated cells of this isolate. Although the exact role of these proteins in biofilm and agar surface-associated growth is not clear, their diverse functions, viz., cellular attachment, motility and the export of proteins, toxins, polysaccharides, antimicrobial agents and other toxic compounds, may be essential for healthy biofilm growth.

Bacterial biofilms are associated with increased resistance towards antimicrobial agents (Donlan, 2002; Huq *et al*., 2008; Johnson, 2007; Sauer and Camper, 2001; Webb *et al*., 2003; Whiteley *et al*., 2001). Induction of multi-drug resistance efflux pumps, such as the systems identified in the sessile growth phases of *F. johnsoniae*-like isolate YO12, may partly explain planktonic multidrug resistance phenotypes of *F. johnsoniae*-like isolates (Basson *et al*., 2008) as well as biofilm-associated multidrug resistance observed in many aquatic pathogens and pathogenic bacteria (Bernardet *et al*., 2006; Bernardet and Nagakawa, 2006; Kerry *et al*., 1996; Miranda *et al*., 2003; Sauer and Camper, 2001; Vila *et al*., 2007; Whiteley *et al*., 2001). Examination of the increased expression of efflux pumps conferring multi-drug resistance in biofilm-associated *Flavobacterium* spp. would establish a novel understanding of antimicrobial resistance in these organisms and requires further investigation.

Cell membrane protein components other than transporters or efflux systems that play an important role in polysaccharide and EPS excretion include lipoproteins (Haft *et al*., 2006). Lipoproteins, such as the TPR-repeat lipoprotein identified in the OMP fraction of isolate YO64 (Chapter 3), are often associated with bacteria capable of polysaccharide and EPS production and biofilm formation (Haft *et al*., 2006; Norton *et al*., 2008). The abundance of these proteins identified by SSH in the biofilm and agar surface-associated phases of isolates YO12 and YO64 may indicate a possible function in protein assemblage of membrane components required for EPS production, cellular attachment and/or biofilm formation.

The presence of putative proteins involved in polysaccharide biosynthesis/ excretion in the two sessile growth phases strongly suggests a role for polysaccharides as part of EPS required for aggregation and surface colonization in these *F. johnsoniae*-like isolates. Differential expression, with possible up-regulation, of a putative SprD protein in isolate YO64 biofilm cells may indicate a role for the spreading ability and/or motility apparatus in attachment and/or biofilm formation of *F. johnsoniae*-like isolates.

Transcription regulatory systems and components of other gene regulatory systems were identified in both the biofilm and agar surface-associated phases of the *F. johnsoniae*-like isolates YO12 and YO64, respectively, using SSH. Interestingly, none of the sigma regulatory proteins were identified in the present study. This may be ascribed to the highly unstable nature of mRNA for the protein components, as observed for the global second messenger c-di-GMP regulatory proteins, diguanylate cyclases and phosphodiesterases, involved in c-di-GMP turnover (Simm *et al*., 2004). An intensive screen of cloned insert sequences obtained by SSH and/or inactivation/expression studies may be necessary to detect components of other possible regulatory systems, such as the sigma regulatory system and/or Spo0A-related system required for biofilm formation in *B. subtilis* (Chapter 3). Inactivation/expression studies and induction of uracil-related mutations may verify the presence of a uracil-related regulatory system (Ueda *et al*., 2009).

The SSH data generated has demonstrated substantial evidence to support the concept of growth-specific physiological changes that occur in sessile *F. johnsoniae*-like isolates. Differential gene expression and/or unique gene expression occurs in surface-associated *F. johnsoniae*-like cells, providing evidence of biofilm-specific phenotypes. The data also provides, in our opinion, sufficient evidence to suggest that changes in cell morphology of surface-associated *F. johnsoniae*-like isolates resulted from alteration in the composition of the outer membrane. As observed for 2-D/MS analysis, the differences observed in gene expression between biofilm and agar surface-associated growth of the *F. johnsoniae*-like isolates may be the result of attachment to different (biotic and abiotic) surfaces, resulting in altered gene expression.

CHAPTER FIVE

CONCLUDING REMARKS

5.1. THE RESEARCH IN PERSPECTIVE

In the present study, the association of phenotypic and genotypic characteristics with biofilm formation of *Flavobacterium johnsoniae*-like isolates obtained from a variety of fish host species and biofilm growth in South African aquaculture systems was investigated. Differential gene expression between planktonic, biofilm and agar surface-associated *F. johnsoniae*-like cells was assessed using a combination of 2-D gel electrophoresis and SSH. Components of differential gene expression in planktonic, biofilm and agar surface-associated *F. johnsoniae*-like cells were also identified. This is the first report of differential expression of genes and gene products during transition between the planktonic and biofilm phases of growth of *F. johnsoniae*-like isolates.

Motility, chitin degradation, ECC production and proteolytic abilities of *F. johnsoniae*-like isolates and biofilm formation seem to be antagonistic properties (Basson *et al.*, 2008). The biosynthesis of ECC may vary during the course of biofilm formation (Sauer *et al.*, 2002). Besides forming part of the capsular material, which may mask cell surface components, such as motility structures required for attachment, ECC may form part of EPS, which may be subject to differential gene expression during the course of *F. johnsoniae*-like biofilm formation. This may explain the significant negative correlations observed between motility and/or ECC production and 24 h biofilm cultures of *F. johnsoniae*-like isolates, respectively. ECC and EPS biosynthesis may be required for autoaggregation during microcolony formation and/or the complex structure of mature *F. johnsoniae*-like biofilms and may be less important in the younger, 24 h biofilm community. This would explain the identification of polysaccharide biosynthesis genes/proteins (putative polysaccharide biosynthesis protein, glycoside hydrolase, alginate lyase, and SusD homologue)

using differential expression experiments. Specific intraspecies interactions, such as autoaggregation, mediated through the production of ECC may give *F. johnsoniae*-like isolates a competitive advantage over other bacterial species in the aquaculture environment.

Many pathogenic bacteria display phase variation, which allow them to switch between a non-pathogenic and a pathogenic lifestyle (Beyhan *et al*., 2007; Kiem *et al*., 2004; Kunttu *et al*., 2009; Overweg *et al*., 2000). *Vibrio cholerae* undergoes phenotypic variation, thereby generating the smooth and rugose colonial morphologies (Beyhan *et al*., 2007). The rugose type is associated with pathogenicity and an increased ability to form biofilms on chitinaceous surfaces as part of a survival mechanism outside the human host (Beyhan *et al*., 2007; Lipp *et al*., 2002; Reguera and Kolter, 2005). In *Flavobacterium columnare*, a strong adhesion capacity and virulence were associated with different colonial morphologies, viz., non-rhizoid/flat and rhizoid, respectively (Kunttu *et al*., 2009). The *F. johnsoniae*-like isolates also display a smooth and hazy colonial morphology. Although the hazy colony type is associated with gliding motility, increased ECC production, chitin-degrading ability, virulence and casein proteolytic activity, it is not associated with a strong biofilm-forming capacity on polystyrene. By contrast, *F. johnsoniae*-like isolates displaying the smooth colony type are non-gliding or display a weak gliding ability, produce less ECC, appear less virulent, display a decreased ability to degrade chitin and weak casein proteolytic activity, but a strong biofilm-forming capacity on polystyrene. Hence, physiological phase variation, generating the smooth and hazy colony types, may facilitate the survival of *F. johnsoniae*-like isolates outside the fish host and in the absence of chitinaceous surfaces, and/or a pathogenic lifestyle in the presence of fish hosts and chitin-rich materials, respectively.

Moreover, increased ECC may also be associated with thick capsule of highly virulent *F. johnsoniae*-like isolates, thereby facilitating invasion of the fish host during infection. In *V. cholerae*, the highly virulent rugose colony type is directly associated with the production of the exopolysaccharide VPS. Mutations in the *vps* regulons was shown to influence the pathogenicity of *V. cholerae*

(Beyhan *et al*., 2007). Similarly, increased production of ECC and/or production of specific exopolysaccharides in *F. johnsoniae*-like isolates may be related to the hazy colonial morphology and virulence of these isolates.

Although it was not possible to correlate a specific PFGE type with biofilm-forming capacity, a significant positive correlation was observed between PFGE types and fish host species. RFLP analysis using *Xho*I and PFGE allowed the superior differentiation and discrimination of *F. johnsoniae*-like isolates compared to PCR-RFLP analysis (Flemming *et al*., 2007), and was useful in clonal identification of *F. johnsoniae*-like isolates. Similar to RAPD and REP profiles (Flemming *et al*., 2007), dominant clones were observed in the *F. johnsoniae*-like isolates obtained from rainbow trout using PFGE. Hence, in combination with PCR-based typing methods, PFGE may be used as epidemiologic tool to identify clonal origins of clinically important *F. johnsoniae*-like isolates and may also be useful in the identification of specific *F. johnsoniae*-like strains associated with disease outbreaks in rainbow trout in South Africa.

Proteomic analysis of WCPs by 2-D gel electrophoresis and OMPs by 1-D gel electrophoresis and MS confirmed that *F. johnsoniae*-like isolates undergo physiological changes during transition between the planktonic and biofilm and/or agar surface-associated phases of growth. Qualitative analysis of 2-D WCP maps revealed similar but distinct proteomes for planktonic, biofilm and agar surface-associated *F. johnsoniae*-like YO12 and YO64 cells, respectively, with differentially expressed and uniquely expressed proteins being visible in each of the three different growth phases. Similarly, SSH revealed differential expression of thirty-two identified gene product inserts in the biofilm and agar surface-associated phases of the *F. johnsoniae*-like isolates YO12 and YO64, suggesting that differential regulation of gene expression occurs when these bacteria change from a planktonic to a sessile lifestyle.

This supports the idea of biofilm-specific phenotypes and it also demonstrates that different growth conditions affect *F. johnsoniae*-like gene expression, giving rise to physiologically distinguished communities. Sauer *et al*. (2002) suggested that the *Pseudomonas aeruginosa* biofilm proteome was

dependent on the process of biofilm formation, rather than the biofilm mode of growth. Similarly, different components of *F. johnsoniae*-like isolates may be induced/involved in the attachment processes and biofilm development on different (biotic and abiotic) surfaces. However, it is important to keep in mind that the proteomes representing each of the planktonic, biofilm and agar surface-associated growth phases of *F. johnsoniae*-like isolates are a snapshot of the physiological state of the cells at a specific point in time (Gygi *et al*., 1999) and could differ considerably by alteration of various parameters, e.g., culture medium, incubation temperature, incubation time, etc.

The putative proteins differentially and/or uniquely expressed in the biofilm and agar surface-associated phases of *F. johnsoniae*-like isolates identified by 2-D/MS were classified into functional categories of adaptation/protection, metabolic processes, membrane/transport/motility and transcription/translation. Among these were putative proteins involved in osmotic and oxidative stress, EPS biosynthesis, adherence, amino acid and aerobic metabolism, efflux systems, as well as putative transcriptional and signal transduction regulators. The protein products of differentially expressed genes identified by SSH in the biofilm and agar surface-associated phases of *F. johnsoniae*-like isolates were also categorized as being involved in adaptation/protection, metabolic processes, membrane/transport/motility and transcription/translation. The putative proteins identified by SSH were also involved in osmotic and oxidative stress, EPS biosynthesis, adherence, amino acid and central metabolism, efflux systems, as well as putative transcriptional regulators. Similar findings were made by Sauer *et al*. (2002) and Prigent-Combaret *et al*. (1999) for *P. aeruginosa* and *E. coli* biofilm cells, respectively. Furthermore, Prigent-Combaret *et al*. (1999) demonstrated that the biofilm mode of growth induced expression of genes involved in EPS biosynthesis, osmoregulation, oxidative stress and cell-to-cell signaling, while those involved in motility and amino acid metabolism were down-regulated. Sauer and Camper (2001) also demonstrated the up-regulation of genes and gene products involved in EPS biosynthesis in *Pseudomonas putida* biofilm cells, while those associated with motility and amino acid metabolism

were differentially expressed and down-regulated, respectively. In the present study, putative proteins involved in EPS biosynthesis, adherence, transport, osmoregulation, oxidative stress, transcriptional regulation, signal transduction, and amino acid metabolism were demonstrated to be up-regulated and/or uniquely expressed in the biofilm and agar surface-associated phases of *F. johnsoniae*-like isolates. Besides the need for cell surface components, EPS and signal transduction systems required for attachment and/or adhesion processes and biofilm development, biofilm cells encounter conditions of osmotic stress, oxygen limitation and competition in the base layers of biofilm growth, which may induce stress response mechanisms as observed for *F. johnsoniae*-like biofilm cells.

It is apparent that a variety of metabolic changes occurred in *F. johnsoniae*-like biofilm and agar surface-associated cells, compared to their planktonic counterparts. Sauer and Camper (2001) also reported a variety of metabolic changes, including changes in carbon and energy metabolism, in biofilm cells of *P. putida*. This may be related to the physiologically defined gradients of cell growth within the biofilm, which lead to a heterogeneous population of cells in different metabolic states (Beloin and Ghigo, 2005; Ghigo, 2003).

None of the known motility genes of *F. johnsoniae* were identified using 2-D/MS and SSH in the present study. Although motility structures, such as flagella and pili, are often associated with initial attachment of bacteria, the production of motility components is often down-regulated and/or suppressed during biofilm development (Donlan, 2002; Prigent-Combaret *et al.*, 1999; Sauer *et al.*, 2002). The expression of gliding motility genes in *F. johnsoniae*-like isolates was down-regulated and/or suppressed after attachment, which may explain the absence of these components in five day biofilm and agar surface-associated cultures. Further work will be necessary to determine whether induction of proteins functional in gliding motility affects attachment and biofilm formation in these *F. johnsoniae*-like isolates.

Biofilm formation is believed to be dependent or at least partially dependent on quorum sensing and other signal transduction systems (Donlan, 2002; Sauer and Camper, 2001). Although possible signal transduction regulatory proteins (putative uridylate kinase, Spo0B-related protein, and SusD homologue) as well as transcriptional regulators (putative thiol:disulphide interchange protein, translation elongation factor G and AsnC family transcriptional regulator) were identified, none of the RpoD (σ^{70}), RpoN (σ^{54}), and/or extracellular function (ECF) sigma factor regulatory proteins associated with *F. johnsoniae* (McBride *et al*., 2009) were identified in this study.

Although adjacent to ECF sigma factor genes, the *susD*-like genes encode proteins that lack an extra N-terminal signaling domain (present in SusC-like proteins), which is thought to interact with anti-sigma factors during cell signaling (McBride *et al*., 2009). However, *susD*-like genes are located on polysaccharide utilization loci (PULs), which contain genes encoding regulatory proteins other than sigma factors, involved in two-component regulatory systems (McBride *et al*., 2009). These regulatory systems are involved in the utilization of soluble and insoluble polysaccharides. The mechanism suggested for efficient chitin utilization by SusD-like (as well as SusC-like) proteins involve the binding of cell surface proteins to chitinaceous substrates (McBride *et al*., 2009). Like the *vpsT* and *vpsR* regulons in *V. cholerae* (Beyhan *et al*., 2007), the *susD*-like and *susC*-like PULs may be involved in the phase variation between the smooth and hazy colonial morphology types of *F. johnsoniae*-like isolates. Similar to the rugose type *V. cholerae* cells, hazy type *F. johnsoniae*-like isolates may display increased adherence to and biofilm formation on chitinaceous surfaces (a *susC*-like and *susD*-like regulated process) in aquatic and soil environments as part of virulence and/or survival. By contrast, smooth type *F. johnsoniae*-like isolates may display increased adherence to and biofilm formation on abiotic surfaces, facilitating the long term survival of these isolates in nutritionally deprived environments.

Identification of the abundant cell surface proteins, i.e., putative lipoproteins, adhesins, porins and efflux pumps, in biofilm and agar surface-

associated *F. johnsoniae*-like cells was not surprising, since alteration in cell envelope proteins seems to go hand in hand with biofilm formation and surface-associated growth (Pitta *et al*., 1993; Rivas *et al*., 2008; Van Houdt and Michiels, 2005). In the present study, the first description of a putative PEP-CTERM family TPR-repeat lipoprotein in *F. johnsoniae*-like isolates and *Flavobacterium* spp. was reported. According to our knowledge, this is also the first description of a PEP-CTERM family protein in a pathogenic bacterium capable of causing disease in farmed fish.

Although porins and transporter proteins often facilitate bacterial attachment to a suitable substratum (Otto *et al*., 2001; Sauer and Camper, 2001; Seyer *et al*., 2005; Van Houdt and Michiels, 2005), these proteins are mainly involved in the removal of superfluous waste materials and other harmful substances, including antimicrobial agents (Donlan, 2002; Huq *et al*., 2008; Johnson, 2007; Kvist *et al*., 2008; Sauer and Camper, 2001; Webb *et al*., 2003; Whiteley *et al*., 2001). Biofilm cells are often associated with increased antibiotic resistance (Donlan, 2002; Huq *et al*., 2008; Johnson, 2007; Kvist *et al*., 2008; Sauer and Camper, 2001; Webb *et al*., 2003; Whiteley *et al*., 2001). Antibiotic-mediated resistance in biofilm cells may be influenced by multiple factors, including growth-arrested cells, reduced penetration and binding by EPS, as well as the up-regulation of efflux systems, such as those identified by 2-D/MS and SSH in the present study. Kvist *et al*. (2008) observed that efflux systems were highly active in biofilm cells, which also seemed to be the case for biofilm cells of the *F. johnsoniae*-like isolates, especially isolate YO12. *F. johnsoniae* is predicted to harbour ABC-type antibacterial peptide transport systems which are regulated by σ^{54} (McBride *et al*., 2009). This suggests a role for efflux systems and sigma factors in the antimicrobial resistance of *F. johnsoniae*. Hence, the up-regulation and/or unique expression of such efflux systems in the biofilm mode of growth, which may be sigma-regulated, in *F. johnsoniae*-like isolates and other *Flavobacterium* spp. may be related to multidrug resistance observed for pathogenic strains isolated from the aquatic environment (Basson *et al*., 2008;

Bernardet and Bowman, 2006; Bernardet *et al*., 2006; Bernardet and Nagakawa, 2006).

Treatment and control of biofilm-associated bacteria remains a problem in the clinical field and industrial environment. Due to multidrug-resistant biofilm cells, alternative methods of treatment for biofilm-related infections to antibiotics may be of value in the clinical field. Kvist *et al*. (2008) demonstrated the reduction of biofilm growth with efflux pump inhibitors (EPIs), which in combination could eliminate biofilm growth completely. Additionally, EPIs were also shown to block antibiotic tolerance of biofilm cells (Kvist *et al*., 2008). Izano *et al*. (2007) demonstrated the use of glycoside hydrolases to detach biofilm growth of *Aggregatibacter actinomycetemcomitans* and sensitize biofilm cells to killing by SDS treatment and other antimicrobial agents. The use of EPIs and polysaccharide-degrading enzymes, such as the putative glycoside hydrolase and alginate lyase identified in the present study, may effectively abolish, prevent and/or lead to dispersal of *F. johnsoniae*-like biofilm cells. These could serve as alternative methods of treatment and control of *F. johnsoniae*-like persistence in aquaculture systems, as well as other aquatic pathogens capable of biofilm formation.

In the present study, 2-D gel electrophoresis was used successfully to identify differences in planktonic, biofilm and agar surface-associated proteomes of *F. johnsoniae*-like isolates. SSH on the other hand, provided in-depth insight into differentially expressed genes of *F. johnsoniae*-like isolates in the biofilm and agar surface-associated growth phases. Furthermore, a combination of 2-D/MS and SSH was used to identify putative functional genes and gene products in biofilm and surface-associated *F. johnsoniae*-like cells.

Few, if any, relevant functional biofilm genes are known in the *F. johnsoniae*-like isolates and *F. johnsoniae*. The genes identified here displayed similarities to components of bacterial adhesion/attachment, biofilm development, gene regulatory systems, antibiotic resistance, and virulence factors. This study has created the foundation to identify *F. johnsoniae*-like genes specifically involved in biofilm formation. Additionally, identification of these components

may lead to the effective control of the *F. johnsoniae*-like biofilm lifestyle, which may have important clinical applications.

It is clear that although a trend of similar functionality in biofilm genes was observed, there is no universal gene-expression pattern within *F. johnsoniae*-like and/or bacterial biofilms. However, the use of a combination of proteomic and transcriptomic techniques, such as 2-D gel electrophoresis and SSH, may improve our capacity to decipher and understand biofilms and other complex forms of microbial growth.

Future work will entail the identification of specific genes involved in the different biofilm processes of *F. johnsoniae*-like isolates. This will involve inactivation/expression studies to determine the role of potential candidate genes for biofilm formation by *F. johnsoniae*-like isolates.

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APPENDIX 1

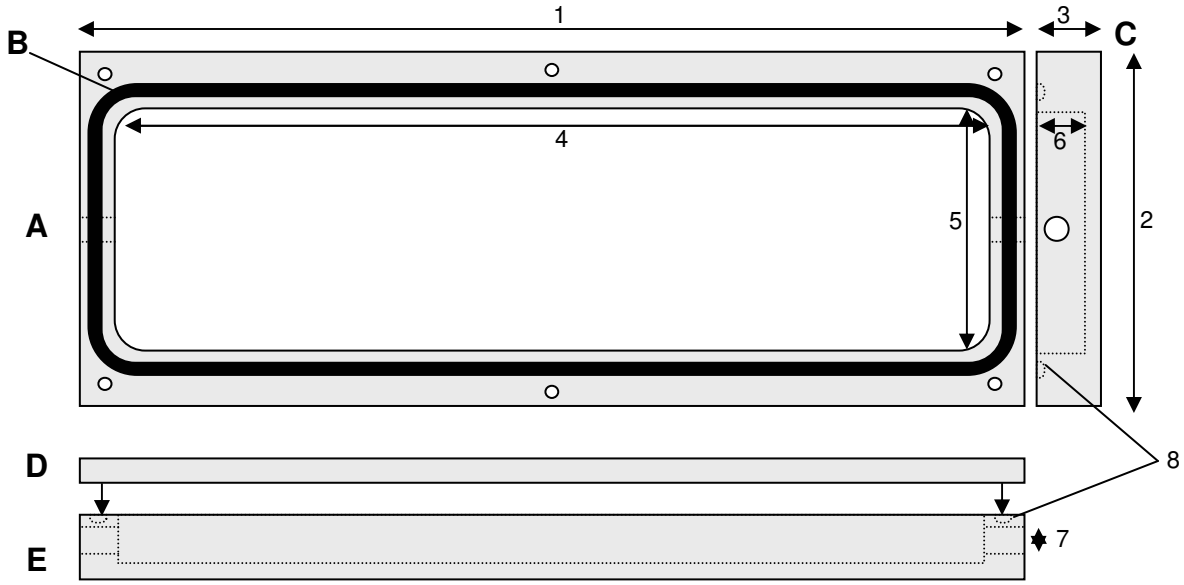

FIG. 1A. Schematic diagram of Perspex flow cell chamber used for biofilm growth of *F. johnsoniae*-like isolates: top view (A) with O-ring (#7, 2 mm) (B), vertical side view (C), lid horizontal side view (D), and horizontal side view (E). Flow cell dimensions: total length (1) = 110 mm; total width (2) = 50 mm; total height (3) = 10 mm; channel length (4) = 100 mm; channel width (5) = 30 mm; channel depth (6) = 6 mm; tube fitting radius (7) = 3 mm; O-ring groove (8) = 2 mm wide $\times$ 1.5 mm deep.

APPENDIX 2

TABLE 2A. Whole cell protein profiles of *F. johnsoniae*-like isolates grown in the planktonic phase

Isolate	YO12	YO15	YO19	YO34	YO45	YO51	YO53	YO59	YO60	YO64	YO66
No. of bands	30	29	30	31	30	25	20	21	29	34	31
Size (kDa)	179.3	179.3			179.3				179.3	179.3	
			150	150		150		150			150
	145	145	145	145	145					145	145
						139.5					
						130					
	126	126	126	126	126					126	126
							117				
										110	
			105	105	105			105	105	105	105
	102	102									
			101	101	101				101	101	101
			100	100		100	100			100	
							97				
			96	96				96		96	96
						95					
	93	93			93		93		93		
						89					
								88.3			
	86	86	86	86	86		86		86	86	86
								85.6			
									84.5		
	84	84				84		84			
	82	82			82		82		82		
			80	80		80		80		80	80
						78					
	77	77			77		77		77		
								75.5			
	73	73	73	73	73	73	73	73	73	73	73
			69	69		69				69	
	68	68			68	68	68		68		68
			67.3	67.3				67.3		67.3	
			64							64	64
	62	62			62	62			62		
			61							61	
	60	60	60	60	60		60	60	60		60
										59	59
	56	56	56	56	56	56	56	56	56		
									55		55
				53.7						53.7	
	50	50	50	50	50	50	50	50	50	50	50
				47	47	47			47	47	47
	46	46	46		46			46	46		
				45		45				45	45
	44	44	44	44	44	44	44	44	44	44	44
	42	42	42	42			42			42	42
	41				41	41			41		
			40	40						40	40
			39.3	39.3						39.3	39.3
	39	39			39				39		
						38.6					
	37	37	37	37	37				37	37	37
						36.7					
										36.5	
			36	36				36			36
	35	35			35				35		
	34.7	34.7			34.7				34.7		
								34.5			
	34	34			34		34		34		
										33.6	33.6
	33.5	33.5			33.5	33.5	33.5				
			33	33				33		33	33
	32	32			32	32		32			
			31.7	31.7			31.7			31.7	31.7
	31	31	31	31	31	31	31	31	31	31	31
			30	30					30	30	30
	29	29	29	29	29	29	29	29	29	29	29
			28	28						28	28
	27	27		27	27		27		27	27	27
	25	25			25				25		

TABLE 2B. Whole cell protein profiles of *F. johnsoniae*-like isolates grown in the biofilm phase

Isolate	YO12	YO15	YO19	YO34	YO45	YO51	YO53	YO59	YO60	YO64	YO66
No.of bands	26	30	41	41	28	37	30	39	34	31	38
Sizes (kDa)					179.3	179.3				179.3	
			146.5	146.5	146.5	146.5					146.5
					143	143					
			134.5	134.5		134.5					134.5
			126	126		126					126
			119	119		119					119
								110	110	111	110
										106	
			105	105	105						
	102				102				102		
		101	101	101			101	101		101	101
						100					
			97	97		97		97			97
			96	96	96			96	96	96	96
									95	95	
						94					
	93		93	93				93			93
		92					92				
						89					
		88.5					88.5				
								87			87
	86				86						
						85					
		84.5				84.5	84.5	84.5	84.5	84.5	84.5
			84	84				84	84	84	84
					83						
	82	82				82	82				
			81.4	81.4				81.4	81.4	81.4	81.4
		81					81				
			80.6	80.6		80.6				80.6	80.6
					80						
			79	79							
			76	76					76		
		74.5					74.5	74.5			74.5
			73	73		73		73		73	73
	70	70	70	70	70	70	70	70	70	70	70
			69	69	69	69					
	68	68					68		68		68
		67			67	67	67		67		
			66.5	66.5		66.5		66.5		66.5	66.5
						63		63	63	63	63
	61	61	61	61			61	61	61		
	60	60	60	60	60	60	60	60		60	60
	59	59	59	59		59	59		59		59
										58.5	
								58	58		
	56	56	56	56	56		56	56	56		
			54	54						55	55
								54	54		
											53
			52	52	52	52		52		52	
	51	51					51		51		
			50	50	50	50			50		
	49	49					49		49	49	
								48			
			47	47							47
						46					
	45.5	45.5					45.5		45.5		
	44	44	44	44	44	44	44	44	44	44	44
	41.5	41.5	41.5	41.5			41.5	41.5			41.5
					40	40			40		
	39.3	39.3	39.3	39.3	39.3		39.3	39.3	39.3	39.3	39.3
	37	37	37	37	37		37	37	37	37	37
						36.5		36.5	36.5		
			36	36				36		36	36
						35.5					
	35	35			35		35	35			
								34.5		34.5	34.5
									34		
						33.5		33.5		33.5	33.5
	33	33	33	33	33	33	33		33		
			32.5	32.5				32.5		32.5	32.5
	31	31	31	31		31	31	31	31	31	31
					30.5	30.5					
	29.5	29.5	29.5	29.5	29.5		29.5	29.5	29.5	29.5	29.5
			29.3	29.3				29.3	29.3		
	29	29	29	29	29	29	29	29	29	29	29
	28	28					28	28			
			27.5	27.5				27.5			
	27	27	27	27	27	27	27		27	27	27
			26.5	26.5				26.5			
	26	26			26	26	26				
	25	25	25	25	25	25	25	25	25	25	25

TABLE 2C. Whole cell protein profiles of *F. johnsoniae*-like isolates grown in the agar surface-associated phase

Isolate	YO12	YO15	YO19	YO34	YO45	YO51	YO53	YO59	YO60	YO64	YO66
No. of bands	20	20	27	27	21	31	22	26	28	31	31
Sizes (kDa)	179.3	179.3			179.3		179.3	179.3		179.3	
					159						
			150	150							150
			145	145					145		145
									130		
			126	126						126	126
						124					
	113	113	113	113	113		113	113		113	113
						112			112		
						110			110		110
						107				107	107
			105	105		105			105		
	102	102			102		102	102			
			101	101					101	101	101
						100					
			95	95		95					
	93	93			93		93		93	93	93
						89					
	86	86	86	86	86	86	86	86		86	86
									84.5		
								84			
						82		82	82		
			80	80		80				80	80
						78		78			
	73	73	73	73		73					73
							70	70	70	70	70
	68	68	68	68	68	68	68	68	68	68	68
									67		
			66	66		66	66	66		66	66
	62	62			62	62	62		62		
	60	60			60	60	60	60		60	
			57	57		57			57	57	57
	56	56			56				56		56
										52	
			50	50		50			50	50	
	46	46	46	46	46	46	46	46	46	46	46
	44	44	44	44	44	44	44	44	44	44	44
						42		42		42	42
	40	40			40	40	40	40		40	40
			39.5	39.5							
						39.3		39.3		39.3	39.3
									38		
								37.3			
	37	37	37	37			37		37	37	37
								36		36	
									35.7		
						35.5		35.5			
			35	35			35	35		35	35
	34.5	34.5			34.5	34.5			34.5		
			34	34		34				34	34
	33.5	33.5			33.5		33.5		33.5		
			33	33				33		33	33
	32	32			32		32		32		
			31.7	31.7						31.7	31.7
					31.5		31.5				
	31	31	31	31	31	31	31	31	31	31	31
			30	30	30		30	30	30	30	30
			28.5	28.5				28.5		28.5	28.5
	27	27			27	27	27				
			26.7	26.7				26.7	26.7	26.7	26.7
						26					
	25	25	25	25	25		25	25	25	25	25

TABLE 2D. Outer membrane protein profiles of *F. johnsoniae*-like isolates grown in the planktonic phase

Isolate	YO12	YO15	YO19	YO34	YO45	YO51	YO53	YO59	YO60	YO64	YO66
No. of bands	18	22	18	16	20	18	14	12	8	23	20
Sizes (kDa)	150	150			150					150	150
			149								
	135									135	135
		130	130		130						
						125					
										120	
				110							
									109	109	109
			104	104		104					
					103				103	103	103
	101	101									
			100	100		100					
					99	99					
	95	95									
			92	92		92	92			92	92
	90	90									
	86										
		85	85	85	85	85	85	85	85	85	85
	80	80	80	80	80	80	80	80	80	80	80
			73	73					73	73	
	72	72				72		72			72
											71
					70		70		70	70	
	68	68									
											67
						66	66	66		66	
			65		65						65
				63						63	63
			62								
		60									
										57	
		56									56
		55			55	55					
				54						54	54
		50			50						
								49		49	49
			48								
	47	47			47	47					
	45	45	45	45	45	45	45	45	45	45	45
							42	42		42	
						41					
				40			40	40			40
	39.5	39.5	39.5		39.5					39.5	
	37	37					37				
					36.7	36.7				36.7	
						36					
			35.5								
	35	35			35		35				
	33.5	33.5			33.5	33.5	33.5		33.5		
			33	33				33		33	33
						30					
	29.9	29.9			29.9		29.9	29.9		29.9	29.9
			29	29	29			29		29	
			28.5	28.5							
	28	28			28		28				
						27.9					
			27.5	27.5				27.5		27.5	27.5
	27.3	27.3			27.3		27.3				
				26							

TABLE 2E. Outer membrane protein profiles of *F. johnsoniae*-like isolates grown in the biofilm phase

Isolate	YO12	YO15	YO19	YO34	YO45	YO51	YO53	YO59	YO60	YO64	YO66
No. of bands	13	18	17	20	11	15	7	14	23	20	11
Sizes (kDa)	230										
									220		220
			200	200							
					190		190		190	190	
						180					
			175	175					175		175
										170	170
	160		160	160							
									150		
			149	149	149	149	149			149	
									145		
									133		
			130	130							
								127			
			125	125							
									120		
			115	115							
										112	112
		110	110	110		110		110	110		
	106		106	106	106	106				106	106
							105		105		
		104				104		104			
									101		
	100	100	100	100			100			100	100
								99	99		
										94	
		90	90	90	90	90					
	87									87	
		85				85			85	85	
				84							
						80				80	
	78	78									
									76	76	76
		73		73		73		73			
	69	69									
		68									
				65							
								63	63	63	
						62					
											58
	55	55			55	55					
									53		
		49			49			49		49	
	48	48			48		48	48	48		48
			47	47						47	
	45	45							45	45	
						44.5	44.5	44.5			
		42			42						
			41	41							
	40	40	40	40	40			40			
								38			
									37.7		37.7
										37	
	36										
									35		
						34					
	33.5	33.5			33.5	33.5	33.5		33.5		
			33	33	33			33		33	33
		32	32	32					32		
		29.5								29.5	
			29	29		29		29			
									28	28	
								27			
									26	26	

TABLE 2F. Outer membrane protein profiles of *F. johnsoniae*-like isolates grown in the agar surface-associated phase

Isolate	YO12	YO15	YO19	YO34	YO45	YO51	YO53	YO59	YO60	YO64	YO66
No. of bands	8	8	23	17	9	17	6	16	11	22	14
Sizes (kDa)			220	220				220	220		
						200					200
						185					
			180	180				180	180	180	180
				170							170
			165					165	165	165	165
								130			
			129								129
								112	112	112	112
	110	110		110		110					
						109					
			108		108				108	108	
				106		106					
	105		105	105		105	105	105		105	
	104	104									
										100	100
			95					95			
		90	90								
			85	85						85	
			80	80							
										79	
				76						76	
			75	75				75		75	
			73								
										72	
										69	
			68								
										67	
			65								
										64	64
			57			57		57			
	56	56			56					56	
			55								
									54		54
			48	48						48	48
				47.5							
			47	47						47	
					46						
			45.5	45.5							
	45	45			45	45	45	45	45	45	45
						42	42	42	42		
				41							
			40		40					40	
	39					39		39			39
	37										
						36.5					
		36			36		36				
						35.5					
	33.5	33.5			33.5	33.5	33.5		33.5		
			33	33				33		33	33
								32			32
		31					31				
			30	30	30	30		30	30		
										29.9	
						29			29		
						28					
										27	
			26		26	26		26			