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Effect of physiochemical factors on autoaggregation and adhesion of *Flavobacterium johnsoniae*-like isolates

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Flavobacterium johnsoniae-like isolates are able to form biofilms and have been associated with disease outbreaks in fish. Surface colonization requires microbial adhesion strategies that are mediated by specific surface-associated molecules. Blocking or inhibiting these molecules would facilitate the identification of adhesins involved in surface adherence and development of appropriate anti-adhesion strategies for *F. johnsoniae*-like isolates. Autoaggregation and adhesion assays were performed using *F. johnsoniae*-like isolates and *Flavobacterium* spp. type strains to determine the anti-adhesion effect of heat, proteinase K, glucose, galactose, mannose, and sodium metaperiodate. Majority of the isolates (~85%) displayed decreased autoaggregation following heat treatment, while ~80% and 100% of isolates demonstrated increased autoaggregation following proteinase K and sodium metaperiodate treatments. Although carbohydrate treatments increased autoaggregation indices, they were not significant. Heat, proteinase K, sodium metaperiodate (SMP), glucose and galactose treatments significantly decreased adhesion of flavobacterial isolates to microtitre plates under static conditions, but not under dynamic conditions. Autoaggregation and surface adhesion by *F. johnsoniae*-like isolates appears to be mediated by chemically diverse groups of adhesins and receptors, given the diverse responses observed following treatment of cells. Glycoprotein molecules form part of the complement involved in mediating successful attachment of *Flavobacterium* spp. isolates to surfaces. To prevent attachment and recurring infections by *F. johnsoniae*-like isolates, understanding the type of adhesin-receptor interactions in adhesion is critical. This will allow the development of strategies to disrupt initial attachment and prevent biofilm formation by *Flavobacterium* spp.

Key words: *Flavobacterium* spp.; anti-adhesion; autoaggregation; biofilm

INTRODUCTION

Aquaculture systems provide the ideal habitat for biofilm-forming pathogenic bacteria, with a rich flow of nutrients, close proximity to the host, and a variety of surfaces amenable for bacterial colonization, allowing disease

outbreaks or the recurrence of infection (Karunasagar et al., 1996; Coquet et al., 2002). Biofilm establishment on host tissue or inanimate surfaces also inhibits effectiveness of antimicrobial therapy, protects against host

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Abbreviations: SMP, sodium metaperiodate; EAOA; enriched Anacker and Ordal's agar; EAOB, enriched Anacker and Ordal's broth; OD, optical density; W and S, weak and strong biofilm-forming phenotypes, respectively; HL and MHB, hydrophilic and moderately hydrophobic, respectively.

defense mechanisms and facilitates bacteria communication leading to the expression of virulence determinants (Lavender et al., 2004).

Bacterial adherence, to biotic or abiotic surfaces, is a complex multi-step process that can be subdivided into the stages of attraction, adhesion and aggregation. The ability to coaggregate and autoaggregate have been found to enhance bacterial attachment, with a larger proportion of biofilm strains being able to coaggregate and autoaggregate compared to their planktonic counterparts (Rickard et al., 2004). Autoaggregation is a "selfish" mechanism whereby a strain within the biofilm will express polymers to enhance the integration of genetically identical strains (Rickard et al., 2003a) and may thus enhance the development of freshwater biofilms. Autoaggregation interactions are enhanced by increased hydrophobicity and tend to be stronger than coaggregation (Rickard et al., 2004). Autoaggregation can be highly specific and is typically mediated by interactions between "adhesins" or specific carbohydrate-binding proteins (lectins) found on one cell and complementary saccharide "receptors" borne on another (Rickard et al., 2003a). Many bacterial adhesins are glycoproteins, and specific receptor-ligand (protein-saccharide or protein-protein) interactions mediate the aggregation of bacterial cells that allow for adherence and biofilm formation, since inhibition or reversal of these interactions by saccharide analogs such as lactose and galactose (Rickard et al., 2003b) or enzymatic treatment has been observed. Bacteria possess specific adhesins that allow for attachment to genetically similar cells (autoaggregation) which enables enhanced microcolony expansion, which is an important stage in biofilm development following attachment to surfaces (Rickard et al., 2003a; 2004). Amyloid fibres, which are the major proteinaceous component of community extracellular matrices, often mediate self and non-self-interactions during biofilm formation. These protein scaffolds aid in adhesion to surfaces and tissues, changing surface properties and resistance to diverse environmental challenges (Blanco et al., 2012).

Members of the genus *Flavobacterium* are known to form biofilms (Álvarez et al., 2006; Basson et al., 2008), not only as a stress response but as an adaptation to allow persistence of cells on the outer surface of the fish host during the early stages of infection (Staroscik and Nelson, 2008) or to survive as saprophytes in the fish farm environment (Kunttu et al., 2009). Adhesion to the host's surface is an essential step in the primary colonization of the host by a pathogenic bacterium, and pathogenic *Flavobacterium* spp. are able to adhere to the gill epithelium, scales and fins of fish hosts (Decostere et al., 1999; Møller et al., 2003; Álvarez et al., 2006), via specific or non-specific mechanisms of attachment. This ability to adhere and/or persist on gill tissue or skin can be closely correlated with their virulence potential (Beck et al., 2012). Specific adhesion is mediated through particular molecules on the surface of the bacterium (lectins), binding to receptors present on host tissue,

whereas non-specific adhesion depends on hydrophobic or ionic interactions between certain structures on the surface of the bacterium and the supporting substrate (Ofek and Doyle, 1994).

Adhesion is typically mediated by proteins on the pathogen that bind to displayed carbohydrate structures on the cell surface. Anti-adhesion approaches are being investigated whereby free carbohydrate molecules are used to interfere with bacterial attachment to biotic and/or abiotic surfaces, thus preventing bacterial adhesion and the subsequent infection or colonization (Pieters, 2007). Non-adhering bacteria can then be removed by cleansing mechanisms appropriate to the targeted surfaces. Using this approach, bacteria are not killed but are rendered non-infective, with the additional benefit of reduced selection pressure being exerted, reducing the development of chemical resistance and fewer problems arise from the release of toxic by-products or DNA from dead bacteria (Pieters, 2007). The anti-adhesion approach provides a useful tool for investigating the role of adhesin molecules in autoaggregation and/or initial attachment to surfaces.

The present study thus focused on determining the role of extracellular adhesins on autoaggregation and adhesion using the anti-adhesion approach. Treatment of the cells with protease, sodium metaperiodate (SMP), carbohydrates and heat, respectively, were used to interfere with autoaggregation of planktonic cells and adhesion of *Flavobacterium johnsoniae*-like isolates to microtitre plate surfaces.

MATERIALS AND METHODS

Bacterial isolates

Twelve (12) previously characterized *F. johnsoniae*-like isolates (YO12, YO15, YO19, YO34, YO45, YO51, YO53, YO59, YO60, YO63, YO64, and YO67) isolated from eel, koi-carp and trout were selected for study based on their biofilm-forming abilities (Flemming et al., 2007; Basson et al., 2008). Isolates were divided into two groups (Table 1) based on their colony morphotypes (Flemming et al., 2007), that is, smooth (YO19, YO34, YO51, YO59, YO63, YO64 and YO67) and hazy (YO12, YO15, YO45, YO53 and YO60). The associated motility characteristics, biofilm phenotypes and hydrophobicity of isolates are indicated in Table 1 (Flemming et al., 2007; Basson et al., 2008). Type strains (*F. aquatile* LMG 4008^T, *F. johnsoniae* (a) NCIB 11054^T, *F. johnsoniae* (b) NCIB 11054^T and *F. pectinovorum* NCIB 9059^T) were also included in the study. Study isolates and type strains were cultured and maintained on Enriched Anacker and Ordal's agar (EAOA) plates or in enriched Anacker and Ordal's broth (EAOB).

Autoaggregation and inhibition of autoaggregation

Bacteria were grown in 50 ml EAOB at 26°C with agitation on a flatbed shaker (120 rpm) for 36 h, harvested, washed three times with sterile 0.1 M phosphate buffer (pH 7), and re-suspended in sterile dH₂O to an optical density (OD) of 0.3 at 660 nm. Percentage of autoaggregation was measured by transferring a 1 ml sample of bacterial suspension to a sterile 2 ml cuvette and measuring the OD after 60 min at a wavelength of 660 nm (Basson

Table 1. Colony morphology, motility, biofilm and hydrophobicity characteristics of *Flavobacterium johnsoniae*-like isolates selected for study.

Isolates	Fish host	Colony morphology*	Motility*	Biofilm formation (OD595 nm)*		Surface hydrophobicity*	
YO12	Rainbow trout	Hazy	+	0.13 ±0.02	W	23.6 ±0.03	MHB
YO15	Rainbow trout	Hazy	+	0.12 ±0.01	W	15.4 ±0.02	HL
YO19	Rainbow trout	Smooth	-	0.37 ±0.03	S	9.8 ±0.01	HL
YO34	Rainbow trout	Smooth	-	0.40 ±0.03	S	14.4 ±0.03	HL
YO45	Rainbow trout	Hazy	+	0.11 ±0.01	W	22.6 ±0.02	MHB
YO51	Rainbow trout	Smooth	-	0.12 ±0.02	W	13.0 ±0.02	HL
YO53	Koi	Hazy	+	0.09 ±0.01	W	19.6 ±0.02	HL
YO59	Longfin eel	Smooth	-	1.06 ±0.08	S	10.9 ±0.02	HL
YO60	Longfin eel	Hazy	(+)	0.09 ±0.01	W	20.9 ±0.02	MHB
YO63	Longfin eel	Smooth	-	0.77 ±0.02	S	17.3 ±0.02	HL
YO64	Longfin eel	Smooth	-	0.91 ±0.23	S	14.5 ±0.02	HL
YO67	Biofilm	Smooth	-	0.11 ±0.01	W	16.5 ±0.03	HL

*Colony morphology (hazy or smooth), motility (-, (+), + indicate non-motile, weakly motile and motile, respectively), biofilm (W and S indicate weak and strong biofilm-forming phenotypes, respectively) and hydrophobicity (HL and MHB indicate hydrophilic and moderately hydrophobic, respectively) characteristics of *Flavobacterium johnsoniae*-like isolates (Flemming et al., 2007; Basson et al., 2008).

et al., 2008). The degree of autoaggregation was determined as the percent decrease of optical density after 60 min using the equation:

$$\% \text{ Autoaggregation} = \left[\frac{OD_0 - OD_{60}}{OD_0} \right] \times 100,$$

where, OD₀ refers to the initial OD of the organism measured. Sixty min after the OD₀ was obtained, the cells suspensions were centrifuged at 2 000 rpm for 2 min and the optical density (OD₆₀) determined. Experiments were carried out in triplicate on two separate occasions (Basson et al., 2008).

The inhibitory effects of four treatments on autoaggregation were investigated for bacteria that were grown in 50 ml EAOB at 26°C with agitation on a flatbed shaker (120 rpm) for 36 h, harvested, washed three times with sterile 0.1 M phosphate buffer (pH 7), and resuspended in sterile dH₂O to an OD_{660 nm} of 0.3. For heat treatment, cells were heated at 80°C for 30 min. Protease sensitivity of potential adhesins was tested using a modification of the method described by Rickard et al. (2003b). Proteinase K (Sigma, St. Louis, MO, USA) was added to the standardized cell suspensions to a final concentration of 2 mg/ml, incubated at 37°C for 2 h, followed by centrifugation and washing of the pelleted cells three times in dH₂O. The ability of simple sugars to inhibit auto-aggregation involved filter-sterilized solutions of glucose, galactose, and mannose (Sigma), respectively, being added to the standardized cell suspensions to a final concentration of 50 mM (Malik et al., 2003). Mixtures were then vortexed and incubated for 1 h at room temperature. Cells were also treated with 20 mM SMP (Sigma), incubated at 22°C for 1 h in the dark, with shaking at 150 rpm, followed by washing of pelleted cells three times in dH₂O (Malik et al., 2003). Following each treatment, the OD of the bacterial suspensions was readjusted to 0.3 at a wavelength of 660 nm and their capacity to autoaggregate was assessed (Basson et al., 2008).

Microtitre plate adhesion and inhibition of adhesion

The effects of four treatments on adherence to polystyrene microtitre plate surfaces were investigated, for bacterial isolates which

were harvested from EAOB cultures grown at 26°C for 18 h, washed three times and re-suspended in dH₂O. Cell suspensions were standardized equivalent to a 0.5 McFarland standard. For the first treatment, 100 µl of standardized cell suspension were transferred to 1.5 ml tubes where proteinase K was added to a final concentration of 2 mg/ml (Rickard et al., 2003b). For the carbohydrate-reversal treatments, filter-sterilized solutions of glucose, galactose, and mannose, respectively, were added at final concentrations of 50 mM (Malik et al., 2003) to 100 µl aliquots of standardized cell suspensions. For the third treatment, standardized cell suspensions were heated at 80°C for 30 min (Rickard et al., 2003b). For the fourth treatment, SMP (20 mM) was added to standardized cell suspensions and cells were incubated in the dark at 25°C with shaking at 150 rpm for 60 min (Malik et al., 2003). Protease- and SMP-treated cells were washed and re-suspended in phosphate-buffered saline (PBS, pH 7.2) to a turbidity equivalent to a 0.5 McFarland standard. Wells of sterile, neutral 96-well U-bottomed polystyrene microtiter plates (Greiner Bio-one) were each filled with 90 µl EAOB and 10 µl of each cell suspension, in triplicate (Basson et al., 2008). Negative control wells contained only EAOB, while respective untreated, standardized cell suspensions in EAOB were used as positive controls. Plates were agitated or kept on the bench-top to simulate dynamic and static conditions, respectively, and were incubated at room temperature (~23°C) for 24 h. Contents of each well were aspirated, washed three times with 250 µl sterile PBS and remaining cells were fixed with 200 µl of methanol for 15 min. After air-drying, wells were stained with 150 µl of 2% Hucker's crystal violet for 5 min. Dye bound to adherent cells was re-solubilized with 150 µl of 33% (v/v) glacial acetic acid and the OD of each well was obtained at 595 nm using an automated Ascent Multiskan RC microtiter plate reader (Thermo Labsystems, Finland). Tests were done in triplicate on two separate occasions and the results averaged (Basson et al., 2008). A measure of efficacy called Percentage reduction was calculated from the blank, control and treated absorbance values (Pitts et al., 2003):

$$\text{Percentage reduction} = \left[\frac{(C-B) - (T-B)}{C-B} \right] \times 100,$$

Figure 1. Inhibition of autoaggregation of *Flavobacterium johnsoniae*-like isolates and *Flavobacterium* spp. type strains following heat (80 °C), proteinase K (2 mg/ml), and sodium metaperiodate (20 mM) treatments. Data represents mean autoaggregation indices $\pm$ SD of three replicates.

Where, B denotes the average absorbance per well for blank wells (no biofilm and no treatment), C denotes the average absorbance per well for control wells (biofilm, no treatment), and T denotes the average absorbance per well for treated wells (biofilm and treatment).

Statistical analyses

Differences in autoagglutination or microtitre plate adhesion between untreated and treated bacteria were determined by paired *t*-tests or Wilcoxon signed rank tests if the homogeneity of variances test failed (SigmaStat V3.5, Systat Software, Inc, CA, USA). Differences were considered significant if $p < 0.05$.

RESULTS

Inhibition of autoaggregation

Physicochemical treatments had varied effects on autoaggregation indices (Figures 1 to 2) either stimulating or inhibiting the autoaggregation of isolates, with isolate-specific behavior being observed. Heat treatment resulted in statistically significant decreased autoaggregation for

87.5% (14/16; $p = 0.014$) of the isolates (Figure 1). Complete inhibition of autoaggregation was observed for seven isolates (Figure 1; YO34, YO45, YO51, YO59, YO63, YO64 and YO67), of which six demonstrated the smooth colony morphotype. Treatment with proteinase K resulted in a statistically significant increase in autoaggregation for 87.5% (14/16; $p = 0.003$) of isolates, relative to untreated cells (Figure 1; Table 1). All isolates displayed a statistically significant increase in autoaggregation following SMP treatment ($p < 0.001$; Figure 1).

None of the sugars used resulted in statistically significant alterations in autoaggregation. However, glucose treatment resulted in an inhibition of autoaggregation for 81.3% (13/16) of isolates (Figure 2; $p = 0.083$). Complete inhibition of autoaggregation by glucose was observed for isolates YO63, YO64 and *F. pectinovorum*. Although galactose treatment resulted in increased autoaggregation indices for four isolates (YO12, YO19, YO34 and YO59), 75% (12/16; $p = 0.447$) of isolates displayed decreased autoaggregation abilities (Figure 2). Complete inhibition of autoaggregation by galactose was observed only for isolates YO63 and YO64 (Figure 2). Following mannose

Figure 2. Inhibition of autoaggregation of *Flavobacterium johnsoniae*-like isolates and *Flavobacterium* spp. type strains following glucose, mannose and galactose (50 mM) treatments. Data represents mean autoaggregation indices $\pm$ SD of three replicates.

treatment, 43.8% (7/16; $p = 0.957$) of isolates displayed decreased autoaggregation (Figure 2), with complete inhibition of autoaggregation being observed for isolates YO63, YO64 and *F. pectinovorum*.

A statistically significant negative correlation was observed between colonial morphology and hydrophobicity of untreated cells ($r = -0.774$, $p = 0.032$). Although a statistically significant negative correlation was observed between colony morphology and autoaggregation of untreated cells ($r = -0.701$, $p = 0.011$), no similar correlations were observed following the respective treatments.

Inhibition of microtitre plate adhesion

Treatment with heat resulted in a statistically significant decrease in adhesion under both dynamic and static conditions (Figure 3). Under static conditions, 93.8% (15/16) of isolates demonstrated a statistically significant decrease in adhesion ($p < 0.001$) following heat treatment (Figure 3b). Although adherence was decreased by proteinase K treatment for 56.3% (9/16) and 68.8%

(11/16) of isolates under dynamic and static conditions (Figure 3), this reduction was statistically significant only under static conditions ($p = 0.008$). Treatment with SMP also resulted in a statistically significant decrease in the adhesion for 87.5% (14/16; $p < 0.001$) of isolates under static conditions (Figure 3b). Isolates with a strong biofilm-forming ability, that is, YO19, YO34 and YO64 displayed significant reduction in adhesion following treatment with heat, proteinase K and SMP under both dynamic and static conditions (Figure 3).

Treatment with sugars resulted in a greater reduction in adhesion under static conditions compared to dynamic conditions (Figure 4). As with the autoaggregation, the anti-adhesion effect of the three sugars under dynamic conditions was not statistically significant. Following glucose treatment, 37.5% (6/16; $p = 0.175$) of isolates displayed reduced adherence under dynamic conditions compared to 81.3% (13/16; $p = 0.021$) of isolates under static conditions (Figure 4). Following galactose treatment, 68.8% (11/16; $p < 0.001$) of isolates displayed statistically significant decreases in adhesion under static conditions (Figure 4b). Although 68.8% (11/16) of isolates

Figure 3. Inhibition of microtitre plate adherence of *Flavobacterium johnsoniae*-like isolates and *Flavobacterium* spp. type strains following heat (80°C), proteinase K (2 mg/ml), and sodium metaperiodate (20 mM) treatments, under A> dynamic and B> static conditions. Data represent the mean $\pm$ SD of three replicates.

also displayed decreased adhesion following mannose treatment under both static and dynamic conditions (Figure 4), neither of these were statistically significant.

Table 2 displays the biofilm reduction efficacy of each treatment, following initial attachment and detachment assays. A positive reduction value indicated inhibition of adhesion, while a negative value indicated increased adhesion (Table 2).

With the exception of glucose and mannose treatments under dynamic conditions, majority of the isolates tested displayed increased biofilm reduction (Table 2). Responses to the treatment appeared to be isolate-specific in some instances, for example, isolates YO45 and YO53, which demonstrated increased adhesion following all treatments under dynamic conditions. Other isolates showed varying combinations of increased

or decreased adhesion following treatments under dynamic and static conditions. Under dynamic conditions, a statistically significant positive correlation was observed between colony morphology and adhesion of galactose-treated cells ($r = 0.663$; $p = 0.019$). However, under static conditions, no correlation was observed between colonial morphotype and/or hydrophobicity and any of the treatments conducted.

Figure 4. Inhibition of microtitre plate adherence of *Flavobacterium johnsoniae*-like isolates and *Flavobacterium* spp. type strains following glucose, mannose and galactose (50 mM) treatments, under A> dynamic and B> static conditions. Data represent the mean $\pm$ SD of three replicates.

DISCUSSION

The ability of *F. johnsoniae*-like isolates to switch between the free-living planktonic state and the biofilm state, contributes to their ability to cause recurring disease outbreaks in fish, resulting in significant morbidity and mortality (Basson et al., 2008). *Flavobacterium psychrophilum* cells are surrounded by polysaccharide-rich capsular material (Møller et al., 2003) which may aid in motility

and adhesion. Attachment to fish tissue and virulence of *Flavobacterium columnare* isolates may be related to the type and quantity of extracellular polysaccharides and proteins present in the capsule or on the bacterial surface. A lectin-like carbohydrate binding substance has been implicated in the adhesion of *F. columnare* to fish tissue (Decostere et al., 1999). A rhamnose-binding lectin has been implicated in *F. columnare* pathogenesis, where agglutination of bacterial

cells is driven by recognition of L-rhamnose/D-galactose residues of the O-antigen of lipopolysaccharides (Beck et al., 2012). However, for *F. johnsoniae*-like isolates, while capsule elucidation is associated with motility and autoaggregation (Flemming, 2010), capsule elucidation and adhesion appear to be antagonistic properties (Basson et al., 2008; Flemming, 2010). Understanding the mechanism of adhesion to abiotic and biotic surfaces is thus critical in order to control *F.*

Table 2. Percentage biofilm reduction following varied physicochemical treatments under dynamic and static conditions.

Parameter	Percent biofilm reduction under dynamic conditions*						Percent biofilm reduction under static conditions*					
	Heat	Proteinase K	SMP	Glucose	Galactose	Mannose	Heat	Proteinase K	SMP	Glucose	Galactose	Mannose
YO12	29.49	42.63	42.04	12.03	35.62	4.67	47.31	90.51	62.87	30.02	45.98	59.93
YO15	61.69	1.81	25.77	0.65	10.50	11.66	45.50	46.20	34.81	29.00	0.00	49.69
YO19	86.44	55.59	74.70	28.43	46.79	14.76	74.73	50.65	75.17	-59.43	42.00	-17.27
YO34	75.34	83.42	82.29	26.62	63.08	48.91	73.81	25.39	49.15	22.00	13.35	-152.19
YO45	-183.63	-142.04	-45.59	-274.06	-32.79	-54.39	-109.00	-276.12	-9.91	13.78	-162.79	-228.85
YO51	18.97	-54.99	41.67	13.39	-27.53	-3.72	70.19	64.72	44.81	57.45	27.09	61.64
YO53	-23.74	-1028.29	-967.36	-432.44	-506.03	-170.13	60.88	19.24	59.99	9.13	-2.85	22.20
YO59	72.97	43.41	49.14	-550.23	6.93	-467.71	79.31	77.16	85.62	71.94	-3.57	58.56
YO60	24.81	-85.76	-85.76	-201.40	-142.13	-265.72	62.72	56.76	47.57	43.84	15.87	85.23
YO63	25.19	66.95	67.02	-15.37	-6.12	58.91	92.84	101.28	86.93	125.47	132.23	115.07
YO64	91.98	89.16	91.57	-23.91	81.16	-15.76	96.80	93.79	100.05	4.01	25.64	20.79
YO67	18.28	-58.58	-18.97	-34.64	-47.86	-29.76	53.63	69.65	86.38	27.86	49.65	20.69
<i>F. aquatile</i>	-60.48	0.77	102.06	-2.18	76.56	14.34	216.13	-221.44	-110.94	-94.36	-177.68	-115.36
<i>F. johnsoniae</i> (a)	85.61	-9.40	40.41	17.09	3.90	9.26	87.62	30.57	87.19	26.80	5.45	23.41
<i>F. johnsoniae</i> (b)	32.97	14.02	-157.41	-317.67	-36.64	48.77	58.57	-45.95	80.53	-50.20	24.66	-22.39
<i>F. pectinovorum</i>	-55.34	-523.69	-84.40	-24.90	62.95	-342.10	54.03	-17.60	83.18	18.35	48.19	95.37

*Biofilm reduction calculated according to Pitts et al. (2003). Treatments used in microtitre plate assays: heat at 80 °C, 2 mg/ml proteinase K, 20 mM sodium metaperiodate, 50 mM glucose, 50 mM mannose and 50 mM galactose.

johnsoniae-like isolate-associated infections.

Given the proteinaceous nature of adhesins, heat and proteinase K are often used in anti-adhesion strategies since heat- and protease-sensitive adhesins may be impacted. Møller et al. (2003) observed that heat treatment at 65°C and proteinase K treatment resulted in inhibition of *F. psychrophilum* hemagglutination with trout erythrocytes. The proteinaceous nature of the hemagglutinating structure suggested the presence of a lectin, which interacted specifically with sialic acid. Högfors-Rönholm and Wiklund (2010) have also observed that the hemolytic activity of *F. psychrophilum* smooth colony variants was impaired following heat and proteolytic treatments. In the present study, heat treatment of *F. johnsoniae*-like

isolates at 80°C resulted in statistically significant decreases in both autoaggregation as well as microtitre plate adhesion, suggesting the involvement of a heat-sensitive protein adhesion or amyloid fibrils. Amyloid adhesins have been identified in natural environment biofilms produced by members of the phylum Bacteroidetes, which comprises members of the genus *Flavobacterium* (Larsen et al., 2007).

Högfors-Rönholm and Wiklund (2010) observed that the hemolytic activity of *F. psychrophilum* rough colony variants was elevated following proteinase K treatments, while no effect on adherence was observed for *F. columnare* cells treated with pronase and trypsin (Decostere et al., 1999). Kunttu et al. (2011) observed significant reduction

in adhesion of all *F. columnare* morphology variants, except for one rough colony variant following proteinase K treatment. In the present study, *F. johnsoniae*-like isolates demonstrated decreased adhesion following proteinase K treatment, while autoaggregation indices increased significantly. The decreased adhesion demonstrated by *F. johnsoniae*-like isolates following heat and proteinase K treatment suggests that protein adhesins present on the flavobacterial cell surface or in the extracellular capsular material play roles in adhesion to abiotic surfaces. While the decreased autoaggregation observed following heat treatment agrees with the lectin-receptor model of autoaggregation, where cell-surface lectins on one cell bind with specific complementary

saccharide receptors expressed on another cell (Decostere et al., 1999; Ofek et al., 2003), proteinase K treatment resulted in a statistically significant increase in autoaggregation. Since many lectins are glycoproteins, they may be insensitive to proteolytic treatment due to protection of the protein component by carbohydrate chains, either the carbohydrate component of lectin itself or from capsular material (Decostere et al., 1999). It could be speculated given the increased autoaggregation observed that proteinase K action on surface-associated proteins could have altered the target protein/s allowing for a better aggregation interaction between cells.

Treatment with SMP, an oxidizing agent which cleaves the C-C bond between vicinal hydroxyl groups, caused a significant reduction in *F. columnare* adhesion (Decostere et al., 1999; Kunttu et al., 2011) and hemagglutination (Decostere et al., 1999) by eliminating the lectin and abolishing adhesion. *F. columnare* chemotaxis, which is correlated with its virulence, was also inhibited by SMP pre-treatment (Klesius et al., 2010). Högfors-Rönholm and Wiklund (2010) observed that for some *F. psychrophilum* rough colony variants, SMP treatment resulted in elevated hemolytic activity. Treatment of *F. johnsoniae*-like isolates with SMP resulted in increased autoaggregation but decreased abiotic adhesion under both dynamic and static conditions. This increased autoaggregation may be attributed to cell surface adhesins and/or receptors which are normally located beneath the capsular layer being exposed to the environment (Ofek and Doyle, 1994), thus facilitating aggregation with corresponding cell surface found on other *F. johnsoniae*-like cells. The decrease in adhesion to the abiotic surface suggests that capsule-associated polysaccharide moieties could potentially play a role in abiotic adhesion processes. The removal or loosening of the capsular material by SMP would eliminate lectins localized in the capsule, thus abolishing adherence.

Shrivastava et al. (2012) have demonstrated that addition of galactose and rhamnose caused dispersion of aggregates formed by over-expression of RemA, a mobile cell surface adhesin involved in gliding motility. While glucose and galactose treatments decreased autoaggregation and mannose increased autoaggregation, these treatments were not statistically significant. Of the three carbohydrates tested in the present study, only glucose and galactose appeared to significantly inhibit abiotic adhesion of study isolates under static conditions (Table 1). Decostere et al. (1999) obtained a 100% decrease in adhesion for *F. columnare* cells following incubation with glucose. This complete inhibition of adhesion was only observed for a single isolate (YO63) in the present study. Glucose may be structurally similar to a saccharide receptor, and may serve as an analogue, occupying the binding site on the complementary adhesin, thus preventing cell to cell adhesion (Pieters, 2007). Decostere et al. (1999) also observed a modest 10-fold decrease in adhesion following *N*-acetyl-glucosa-

mine, galactose, and sucrose pre-incubation of *F. columnare* cells. Similarly, Møller et al. (2003) obtained decreased adherence and hemagglutination inhibition following glucose and galactose treatment of *F. psychrophilum* cells. Mannose exposure of *F. johnsoniae*-like isolates in the present study did not produce significant changes in autoaggregation and/or adherence, as has been observed for *F. columnare* cells (Decostere et al., 1999). Wright et al. (2010) obtained reduced adhesion (60%) of *Burkholderia multivorans* with 5 mM of mannose and 20 mM of galactose. According to Decostere et al. (1999), the varying degrees of inhibition observed following sugar treatments (glucose, *N*-acetyl-glucosamine, galactose and sucrose) suggested the involvement of multiple adhesins localized on the *F. columnare* surface and this could explain the varying levels of inhibition observed in the *F. johnsoniae*-like isolate adhesion assays.

The concentration of simple sugars required to inhibit bacterial attachment are considerably high (Wright et al., 2010). The range of carbohydrates tested in the present study was relatively small compared to similar studies (Decostere et al., 1999; Møller et al., 2003), and the saccharides themselves were relatively simple. Monovalent carbohydrates have a limited affinity for target adhesin proteins that are often multivalent (Pieters, 2007), thus future studies should include a wider range of carbohydrates, such as *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetyl-neuraminic acid, or combinations of physicochemical treatments. Glyconjugates with an affinity to bacterial adhesins could then be designed as potential inhibitors of bacterial attachment, once the adhesin-receptor structures are identified (Pieters, 2007).

Autoaggregation and adhesion of *F. johnsoniae*-like isolates were inhibited following heat treatment, however, SMP and proteinase K appeared to have contrasting effects on autoaggregation and adhesion. This suggests that different surface-associated molecules are associated with the processes of attaching to genetically identical cells and abiotic surfaces. Based on the varied response of isolates to the six physicochemical treatments, the nature of the adhesins involved in autoaggregation and/or adhesion by *F. johnsoniae*-like isolates do not appear to be identical and may involve multiple and/or multivalent adhesins.

Rickard et al. (2000) observed that coaggregation among five aquatic bacteria was mediated by varying lectin-saccharide interactions, since aquatic strains may carry multiple adhesins or receptors or a combination of both on their surfaces. This might be true for members of the genus *Flavobacterium*, given the diversity of responses observed following anti-adhesion treatments, with adhesion molecules containing both carbohydrate and protein moieties (Kunttu et al., 2011). *Flavobacteria* might also utilize a "switch on-off" mechanism which involves environmental control of adhesins or receptors associated with the bacterial surface in direct relation to nutri-

tional and possibly quorum-sensing cues. Given their saprophytic nature (Kunttu et al., 2009), *Flavobacterium* spp. are not only transmitted from dead fish to live fish, but are also capable of surviving long periods in water outside the fish host. Their ability to form aggregates by autoaggregation, coaggregation or adherence to biotic and/or abiotic surfaces (biofilms) might be directly linked to the ability to vary the complement of adhesin-receptors expressed on the cell surface. This would allow persistence in the aquatic environment outside the living host, in decaying fish material, on aquatic surfaces and would allow for recurring outbreaks of disease. It is important, however, to note that abiotic adherence and autoaggregation would not be entirely reliant on cell-surface protein moieties, and other biomolecules may play a role in adhesion to surfaces.

Investigation of the processes involved in *Flavobacterium* spp. biofilm formation is important since these structures play a crucial role in their pathogenicity and may be the key to controlling biofilm formation. An understanding of the specific mechanisms used by *Flavobacterium* spp. to attach to abiotic surfaces, to host tissue or to genetically identical cells, may allow the development of strategies which disrupt initial attachment of cells thus preventing biofilm formation.

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