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1 **Gliding motility and expression of motility-related genes in**

2 **spreading and nonspreading colonies of *Flavobacterium***

3 ***columnare***

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16 **Running head**: Gliding motility in *Flavobacterium columnare*

17 **Key words:** colony type, *Flavobacterium columnare*, gene expression, gliding motility,

18 nutrients, RT-qPCR, T9SS, type IX secretion system

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ABSTRACT

Gliding motility machinery enables moving on surfaces in many species among Bacteroidetes, resulting in bacterial colonies with spreading appearance. The adhesins required for gliding are secreted through a gliding motility –associated protein secretion system known as the type IX secretion system (T9SS). The fish pathogen *Flavobacterium columnare* produces spreading (Rhizoid, Rz; Soft, S) and nonspreading (Rough, R) colony types, of which only the spreading Rz type is virulent. In this study, we explored the spreading behaviour of these colony types by microscopic imaging and measured the expression of genes associated with gliding motility and T9SS (*gldG, gldH, gldL, sprA, sprB, sprE, sprF, sprT* and *porV)* under high and low resource levels. The spreading colony types responded to low resource level by increased colony size. The nonspreading colony type as well as the cells subjected to high nutrient level expressed only moderate cell movements. Yet, low nutrient level provoked more active gliding motility by individual cells and increased biofilm spreading by cooperative gliding. The gene expression survey demonstrated an increased expression level of *sprA* and *sprF* under low nutrient conditions. Surprisingly, the expression of gliding motility genes was not consistently associated with more active spreading behaviour. Our study demonstrates that environmental nutrient level is an important regulator of gliding motility and also the expression of some of the associated genes. Furthermore, our results may help to understand the connections between nutrient concentration, gliding motility and virulence of *F. columnare*.

INTRODUCTION

Gliding motility is a process of bacterial movement on surfaces in several bacterial species in the phylum Bacteroidetes (1). Instead of involvement of flagellae or pili, gliding motility is enabled by complex machinery which has been studied more closely in *Flavobacterium johnsoniae* (for a review of *Flavobacterium* gliding motility, see (2)), a model system for gliding motility. Number of studies on flavobacterial gliding motility have led to identification of several genes involved in motility, including *gldA, gldB, gldD, gldF, gldG, gldH, gldI, gldJ, gldK, gldL, gldM, gldN, sprA, sprB, sprE, sprT* (3-15). Furthermore, a subset of these genes, *gldK, gldL, gldM, gldN, sprA, sprE* and *sprT*, has been found to compose a protein translocation system, designated as type IX secretion system (T9SS) (1, 10). T9SS-related genes are restricted to Bacteroidetes, with no prevalent similarity between

51   the previously described secretion systems (1). T9SS has an important role in secretion of

52   gliding motility adhesins, required for surface contact, but also for secretion of virulence

53   factors (10, 16). For example, periodontal pathogen *Porphyromonas gingivalis* uses T9SS for

54   secretion of its major virulence factors, gingipains and hemagglutinins (10, 16).

55   The exact role of each component in the gliding motility machinery of *F.*

56   *johnsoniae* is not yet fully understood. GldB, GldD, GldH, GldI and GldJ are lipoproteins

57   needed for gliding but their exact functions are not known (4, 5, 7-9). GldA, GldF and GldG

58   form an ABC transporter but its role in gliding is still poorly known (3, 6). *sprB* is a motility

59   adhesin needed for gliding and it is secreted via the T9SS (10). SprB encoding gene is located

60   in operon *sprCDBF* where it is transcribed together with *sprC* and *sprD*, genes coding for

61   proteins that support SprB function, and *sprF* which is needed for successful secretion of

62   SprB (17). In addition, a recently identified secretion-related gene, *porV*, is needed for

63   secretion of chitinase and adhesin RemA in *F. johnsoniae* (14). The mechanisms that control

64   the assembly and activity of gliding motility machinery and T9SS are not known. In *P.*

65   *gingivalis*, a two-component regulative system, consisting of PorX and PorY, regulates the

66   expression of a subset of T9SS genes (10).

67   *F. columnare* is a fish pathogen belonging to phylum Bacteroidetes. *F.*

68   *columnare* carries the majority of the orthologous genes (18) involved in *Flavobacterium*

69   gliding motility and T9SS, which are used for virulence factor secretion and formation of

70   spreading colonies (2, 10). Recently, a transcriptome-wide study in *F. columnare* strain

71   ATCC 49512 demonstrated that genes associated with gliding motility and spreading are

72   located in actively transcribed operons (19). *F. columnare* can form different colony

73   morphotypes, spreading colony types Rz (rhizoid) and S (soft) and nonspreading type R

74   (rough) (20, 21). Spreading colony morphology has been suggested to be essential for *F.*

75   *columnare* virulence (20, 21), and indeed, only the spreading rhizoid Rz type is virulent in the

76   fish host (20-22). Furthermore, changes in nutrient concentration in agar culture changes

77   spreading of *F. columnare* colonies, especially in the virulent Rz type (20). Nutrient

78   availability has also a significant impact on virulence in *F. columnare* as high nutrient level

79   induces higher virulence in the bacteria (23, 24). However, the functionality of gliding

80   motility and T9SS in different *F. columnare* morphotypes is not known, although *gldL*, *gldM*,

81   *gldN*, and *gldH* have been suggested as putative virulence-associated factors in *F. columnare*

82   (25, 26). Here, we explored gliding motility in *F. columnare* spreading (Rz, S) and

83   nonspreading (R) morphotypes under conditions that were expected to induce (low-nutrient)

84   or reduce (high-nutrient) spreading behaviour. Gliding motility and individual cell

85 movements were seen to be more active under low-nutrient conditions. We also performed a

86 RT-qPCR (Reverse Transcription quantitative PCR) assay in order to measure the gene

87 expression of T9SS or gliding motility -associated genes *gldG, gldH, gldL, sprA, sprB, sprE,*

88 *sprF, sprT* and *porV*. Of these genes, *gldL, porV, sprA, sprE* and *sprT* are associated with the

89 T9SS. Increased gene expression in response to low nutrient availability was detected in *sprA*

90 and *sprF*. However, the spreading and nonspreading colony types had different expression

91 profiles under different resource levels which could be an indication of divergent metabolic

92 programs.

93

94 **RESULTS**

95

96 **Nutrient availability regulates colony spreading in Rz and S morphotypes**

97 The morphology of bacterial colonies originating from the same bacterial liquid culture and

98 grown on 0.5xN or 2xN Shieh plates for 2 days was detected (Figure 1). Rz colonies grown

99 on 0.5xN Shieh plate were spreading with increased mean colony size (3.95 cm, S.E. +/-

100 0.42) and production of root-like protrusions typical for Rz morphology (Figures 1 and 2A).

101 Rz colonies grown on 2xN Shieh plates had smaller mean colony size (0.73 cm, S.E. +/-

102 0.04) and root-like structures, if seen, were only moderate (Figures 1 and 2A). Type S

103 responded to changing nutrient availability comparably to Rz (mean colony size 2.4 cm, S.E.

104 +/- 0.23 in 0.5xN and 0.75 cm, S.E. +/- 0.06 in 2xN). However, when grown at lower nutrient

105 conditions (0.5xN Shieh), root-like structures were observed also in S type. These colonies

106 were, nevertheless, distinguishable from Rz colonies by their non-adherent, opaque and moist

107 colony appearance (Figure 1). R type did not remarkably alter the colony size in response to

108 changing nutrient availability (mean colony size 0.7 cm, S.E. +/- 0.35 in 0.5xN and 0.5 cm,

109 S.E. +/- 0.00 in 2xN) and root-like structures were seen under low nutrient conditions only

110 occasionally (Figure 2A).

111 **Growth and biofilm formation in varying nutrient concentrations**

112 The viability of Rz, R and S colony types in 0.5xN and 2xN Shieh medium was measured as

113 maximum optical density (OD_{max}) reached during a 65-hour-cultivation (Figure 2B). All the

114 colony types reached the highest OD_{max} at higher nutrient level (2xN Shieh). The biofilm

115 forming ability was remarkably higher under low nutrient condition (0.5xN Shieh) in the Rz

116 type compared to 2xN Shieh as well as R and S types, which were weaker biofilm producers

117 in both nutrient levels (Figure 2B).

118 **Imaging of bacterial movements**

119 The movements of individual Rz cells grown on 0.5xN and 2xN Shieh agar plates was

120 recorded with a confocal microscope. The movements of Rz cells were comparable with

121 previously described gliding motility of *F. johnsoniae*: the cells glided over surface

122 straightforwardly, occasionally attaching to the surface with one end of the cell, rotating and

123 changing the moving direction (Supplementary Videos 1-2). The gliding speed was slower

124 than that seen in *F. johnsoniae* which was used as reference (data not shown).

125 The movements of individual bacteria growing as a part of forming biofilm were recorded

126 between agar layer and microslide chamber bottom. The colony types Rz and S formed a

127 monolayer on the edges of spreading bacterial biofilm where the cells were organized side by

128 side and glided along the adjacent cells (Figure 3; Supplementary Videos 3-4 and 7-8). The

129 cells formed branching rhizoid-like structures, a few cells wide, (here referred to as

130 microrhizoids) which involved both motile and non-motile cells. In both Rz and S types,

131 more active gliding motility was seen under low nutrient concentration (0.5xN). Colony type

132 R expressed only occasional movements regardless of the nutrient level and cellular

133 organization as a spreading biofilm was not observed (Supplementary Videos 5-6). In order

134 to visualize *F. columnare* colony formation at longer timescale, the growth of Rz colony type

135 on 1x Shieh was recorded during an 8-hour recording (Supplementary Video 9). In the front

136 of the biofilm, the bacteria were characteristically organized in microrhizoids which moved

137 cooperatively towards the spreading direction of the biofilm and seemed also to serve as

138 routes along which other cells were able to glide further and support biofilm expansion.

139 **Sequence analysis and expression of gliding motility associated genes**

140 Genes putatively involved in gliding motility were sequenced from Rz, R and S types of

141 strain *F. columnare* B067. No genetic differences were detected between the colony types in

142 the gene sequences of operons *gldFG* and *gldKLMN,* genes *gldH, sprA, sprC, sprD, sprE,*

143 *sprF, sprT, porV, porX, porY* or in the predicted regulative regions upstream the genes *gldH,*

144 *sprA, sprE, porV* or operons *gldFG*, *gldKLMN* and *sprCDBF*. Expression of genes *gldG,*

145 *gldH, gldL, sprA, sprB, sprE, sprF, sprT* or *porV,* which are putatively involved in *F.*

146 *columnare* gliding motility, was measured in B067 Rz, R and S colony types that had been

147 grown on 0.5x and 2x Shieh agar plates. Of these genes *gldL, sprA, sprE, sprT* and *porV* are

148 associated with the T9SS. Gene expression results were normalized with reference genes

149 *gapdh* and *glyA* which are stably expressed in the current dataset (M value 0.5834 for both

150 genes with variance coefficient of 0.2114 for *gapdh* and 0.2007 for *glyA*). Relative

151 expressions are presented in Figure 4 (for statistics, see Tables 2 and 3). Significant

152 differences between colony types were observed in expression of genes *gldG, gldH, gldL* and

153 *sprE* (Table 2, Figure 4, for pairwise comparisons see Table 3). Nutrient level had a

154 significant effect on *gldL, sprA, sprB* and *sprF* expression. The pairwise comparisons

155 revealed that *sprA* was expressed at significantly higher level in low-nutrient conditions in Rz

156 and R types, and the same pattern was detected in *sprF* expression in R type (Table 3; Figure

157 4). However, significant interaction of colony type and nutrient was detected in *gldH, gldL,*

158 *sprA* and *sprE* (Table 2), indicating that gene expression of colony types may differ between

159 nutrient conditions. Indeed, direct associations between spreading behaviour and gliding

160 motility gene expression were challenging to form as different colony types seemed to

161 respond differently to the nutrient level with motility gene expression. Even though a

162 significant effect of colony type was not observed in either *sprT* or *porV,* a significant

163 difference between Rz and R was observed in *sprT* and between Rz and S in *porV* expression

164 (Table 3).

Proteolytic activity and extracellular secretion in different nutrient concentrations

166 Colony types Rz, R and S were cultivated on 0.5xN and 2xN Shieh plates containing 1.5 %

167 skim milk. Proteolytic activity was observed in each colony variant, seen as formation of a

168 clear degradation zone peripheral to the bacterial growth, but no differences between the

169 colony types were observed and the nutrient concentration did not affect to the proteolytic

170 activity (Supplementary Figure S1). Effect of colony type and nutrient availability on

171 contents of extracellularly secreted products (ECP) was analysed further. Generally, Rz, R

172 and S grown in 0.5xN and 2xN liquid Shieh cultures shared a common overall ECP profile,

173 with some moderate changes between individual protein bands between colony types

174 (Supplementary Figure S2). However, a strong protein band, approximately 13 kD in

175 molecular weight, was detected in Rz type grown in both 0.5xN and 2xN Shieh media. The

176 corresponding band was absent or barely detectable in R or S types.

DISCUSSION

Ability to move towards nutrient sources and the host is essential for the survival of bacteria, and bacterial virulence has been shown to be associated with motility in several bacterial species (e.g. (27-29)). Comparative genomic analysis has revealed that members of Bacteroidetes employ a unique gliding motility machinery and a motility-related secretion system T9SS (1). Cells possessing functional gliding motility system form characteristically spreading colonies (2). *F. columnare* and *F. psychrophilum* are pathogenic in fish, affecting freshwater aquaculture at a global scale (30-32). It has been suggested that T9SS may have a central role in the pathogenesis of these species (20, 33), but so far this connection has remained poorly understood. We imaged gliding motility of spreading and non-spreading colonies of *F. columnare* and measured the expression of genes related to gliding and secretion. Colony types Rz and S responded to decreased nutrient concentration by increasing spreading behaviour, but R type did not have this plastic feature, as reported previously by Laanto *et al*. (20). However, mutations were not found in the studied gliding motility genes, indicating that also other genes may be needed in formation of spreading colonies. Although decrease in nutrients remarkably increased spreading of colonies, it did not correspond to expression of gliding motility genes in a uniform manner. We also found that capacity for extracellular secretion of proteases was maintained in the non-spreading morphologies, which may denote presence of functionally intact secretion systems.

Each colony type was viable both at low and high nutrient conditions when they were cultivated in liquid medium. Even though each colony type succeeded better in high nutrient level (in liquid), they all expressed low colony spreading when they were cultivated under the same nutrient conditions on agar plate. This finding indicates that smaller colonies are not produced on high-nutrient-agar due to unfavourable growth conditions but rather as a result of reduced cell motility. In biofilm measurements, we found that Rz type was the most efficient biofilm producer in the low nutrient treatment. However, S type failed to produce biofilm, although this type increased colony spreading under low nutrient conditions. This indicates that spreading *per se* is not an indicator of biofilm formation. Biofilm formation is a process in which successful adhesion is required in order to attach to a surface (34). As the biofilm formation capacity of S type was comparable to control (growth medium without bacteria) it may be incapable of proper surface adhesion, possibly due to lack of functional cell surface adhesins. Indeed, the colonies of the S type are only moderately adherent (35) and can be easily removed from agar plates compared to the Rz and R colony types.

210 However, capacity for extracellular secretion, gliding and adhesion to other cells
211 demonstrated in the S colony type indicates that different adhesins are needed for surface
212 adhesion and social motility of *F. columnare.* The adhesins required for *F. columnare*
213 attachment to surfaces of different composition (abiotic or biotic) are yet to be identified.

214   Microscopic microrhizoids were observed at the edge of spreading colonies.
215 Importantly, the cells involved in microrhizoids expressed cooperative behaviour in terms of
216 social motility as they glided along neighbouring cells and thereby mediated the spreading of
217 the biofilm. Previously, we have proposed the involvement of social movements in *F.*
218 *columnare* biofilm formation due to coordinated cell organization (22). Indeed, bacterial
219 pathogens are known to cooperate, especially with regards to biofilm formation (see e.g.
220 (36)). Furthermore, high nutrient level reduced gliding behaviour in biofilm. We have
221 recently shown that high environmental nutrient level leads to higher virulence via increased
222 virulence factor expression (23). Therefore, it remains to be resolved whether motility itself is
223 essential for *F. columnare* virulence or if gliding motility and virulence are related solely via
224 a common secretion route for adhesins and virulence factors.

225   Previous studies in *F. johnsoniae* have demonstrated that a mutation in any of
226 the gliding motility genes will result in disruption of the gliding motility machinery and
227 formation of non-spreading colonies (see e.g. (2)). In order to study the genetic background
228 of gliding motility in *F. columnare* spreading and nonspreading colony types, we sequenced
229 genes involved in gliding motility apparatus and T9SS; *gldH*, *sprA*, *sprE*, *sprF*, *sprT*, *porV*
230 and genomic regions spanning *gldFG*, *gldKLMN* and *sprCD*. Surprisingly, these genes were
231 identical between the spreading (Rz and S) and the nonspreading (R) colony types.
232 Furthermore, genetic differences were not found in the predicted regulatory regions.
233 Sequence analysis of other *gld* genes could provide information on differences between the
234 colony types, but it is possible that also other genes are involved in colony spreading. In *F.*
235 *johnsoniae,* for example, *secDF* mutants were incapable for gliding motility and chitin
236 utilization, and produced non-spreading colonies (37). It was hypothesized that SecDF may
237 not be involved in gliding directly, but may have a role in translocation of GldJ (37).
238 Furthermore, transposon mutagenesis revealed that a thiol oxidoreductase-like protein TlpB
239 is associated with gliding motility and virulence in *F. psychrophilum* (38). Thiol
240 oxidoreductases are essential for folding of several proteins, including those related to
241 virulence (39), but their function in *F. columnare* has not been studied.

242   We studied the gene expression of gliding motility or spreading -associated
243 genes in low nutrient (0.5x Shieh) and high nutrient (2x Shieh) agar media.   Colony

244 spreading increased in low nutrient conditions and decreased in high nutrient conditions
245 (Figures 1-2). Expression of *gldG, gldH, gldL* and *sprE* (in pairwise comparison also *sprT*
246 and *porV,* Tables 2 and 3*)* was significantly influenced by colony type, but the highest
247 expressions were often observed in the non-spreading R type. In general, gene expression
248 levels were not consistently associated with the activity of gliding motility. Growth under low
249 nutrient conditions significantly increased expression on *sprA* in Rz and R types, and the
250 expression of *sprF* in the R type. In *F. johnsoniae*, SprA has been identified as a cell surface
251 protein and it has been proposed to serve as link between surface adhesins and gliding motor
252 (12). In that sense, increased production of SprA under low-nutrient level seems reasonable.
253 Previously, it has been found that *F. psychrophilum* GldN expression increases *in vivo* and in
254 iron limited media (40). Indeed, low nutrient conditions induce motility in *F. psychrophilum*
255 and *Vibrio parahaemolyticus* (41, 42), whereas *S. enterica* serovar Typhimurium and
256 *Escherichia coli*, that express swarming motility on solid surfaces, act conversely (43, 44).
257 Combined with the observed phenotypic changes in colony spreading and gliding motility,
258 our findings indicate that environmental nutrients may rather regulate the activity of gliding
259 motility machinery, than the abundance of different machinery components. While qPCR
260 measures the quantity of present mRNA of a target gene (45), bacterial transcripts of secreted
261 proteins may experience post-transcriptional, as well as post-translational processing steps
262 prior to transportation through the cell membranes. Consequently, failure in any of these
263 processes may lead to unsuccessful protein translocation, and further alter the cell surface
264 constitution. Therefore, malfunctions in the T9SS may disrupt secretion and result in
265 accumulation of secreted products in cytoplasm or periplasmic space (46, 47). It is also
266 possible that gliding motility-related genes may be regulated via more complex pathways. In
267 *P. gingivalis*, PorX and PorY form putatively a two-component signal transduction system
268 that regulates the expression of a subset of T9SS genes, including *porT, sov, porK, porL,*
269 *porM* and *porN* (10), that correspond *sprT, sprA, gldK, gldL, gldM* and *gldN* of *F.*
270 *columnare*, respectively. The gene sequences of *F. columnare porX* and *porY* orthologs were
271 found to be identical in different colony types, which implicates that the observed gene
272 expression differences are not a result of a mutation in these genes. Hence, the role of PorX
273 and PorY or other regulatory mechanisms that would direct gliding motility or T9SS activity
274 remain to be studied in more detail. For example, preparation of *porX* and *porY* null mutants
275 and exploring the effect on gene expression could make an important contribution to the
276 understanding of gliding motility and its regulation.

277   All the colony types showed proteolytic activity on high and low nutrient milk

278   agar, indicating that colony spreading is not associated with secretion in this *F. columnare*

279   strain. Previous studies have demonstrated that similarly to disruption of gliding motility,

280   mutations in the gliding motility genes affect *F. johnsoniae* proteinase secretion (10).

281   Therefore, our results indicate that the genes related to gliding motility and T9SS in the

282   studied strain B067 are intact. Furthermore, no considerable differences in proteolytic activity

283   were detected on low and high nutrient milk agar, even though in some cases low nutrient

284   concentration has been shown to decrease protease activity (48). The ECP profiles of Rz, R

285   and S types grown in low and high nutrient conditions did not differ remarkably from each

286   other, except for a ~13 kDa protein band, detected in ECP, which was present only in Rz

287   type. This protein has been connected to the virulent colony type also previously (22), but its

288   role in pathogenicity is unknown. However, it should be noted that ECP profiles were

289   isolated from bacteria grown in liquid media and therefore their ECP profiles may not fully

290   correspond the profiles that may have been obtained from bacteria grown on an agar plate

291   (and which would express gliding motility more vigorously).

292   To conclude, environmental nutrients are important regulators of *F. columnare*

293   gliding motility and the expression of associated genes. Despite the mounting data of

294   *Flavobacterium* gliding motility and T9SS, the knowledge of their regulatory pathways is

295   limited, and how environmental cues contribute to the regulation of these signalling pathways

296   in *F. columnare* remains to be determined. Therefore, it cannot be excluded that differentially

297   spreading morphotypes of *F. columnare* are caused by some other factors that are yet to be

298   identified. Understanding the differences of spreading and non-spreading morphotypes may

299   help to disentangle factors related to gliding motility and virulence in *F. columnare*.

300   **MATERIAL AND METHODS**

301   **Bacterial strains and growth conditions**

302   In all of the experiments of this study, we used three different colony types (Rhizoid Rz,

303   Rough R and Soft S) of *F. columnare* strain B067 (49). The bacterial strain was originally

304   isolated from a trout that has been killed during columnaris disease outbreak at Finnish fish

305   farm (49). The Rough colony type was obtained after exposing the original Rz isolate to

306   bacteriophages (20). The Soft type appeared spontaneously during laboratory culture of the

307   Rz type (22).

308   Bacterial stocks were stored at -80 ˚C in 10 % FCS and 10 % glycerol and

309   revived from the freezer in fresh modified Shieh medium according to Song *et al*. (50) which

310   is referred to as Shieh medium in this study and used as a base of nutrient-modified media. In

311   nutrient-modified media (0.5x or 2x Shieh), all the ingredients of the medium were either

312   halved or doubled. In 0.5xN and 2xN Shieh media, only the concentration of peptone and

313   yeast extract were either halved or doubled, respectively (for detailed compositions of the

314   media, see (23)). After revival from the freezer, liquid bacterial cultures were cultivated at

315   RT/26 ˚C, with agitation of 115/150 rpm for 24-48 h to obtain dense cultures. Cultures were

316   refreshed with fresh 1x Shieh medium and cultivation was continued for 16-24 h. For plate

317   cultures, dense liquid culture was streaked on Shieh agar plate which was incubated at RT for

318   two days.

319   **Colony morphology, growth and biofilm formation in different nutrient conditions**

320   Liquid bacterial cultures of Rz, R and S grown in 1x Shieh were streaked on 0.5xN and 2xN

321   Shieh plates. Plates were incubated for 2 days at RT after which the colony morphology was

322   determined.

323   In order to evaluate the bacterial viability in different nutrient concentrations,

324   1.18×10^7-1.28×10^7 colony forming units of B067 Rz, R and S in total volume of 400 µl

325   0.5xN, 1x or 2xN Shieh (N=8) was cultivated on Honeycomb 2® microplate (Growth Curves

326   Ltd) in Bioscreen C™ spectrophotometer (Growth Curves Ltd) at 26 ˚C. The absorbance

327   (600nm) was measured every five minutes for 65 hours. The viability was estimated as the

328   maximum absorbance recorded during the cultivation.

329   The biofilm formation capacity under various nutrient conditions was

330   determined by cultivating 1.7×10^6–1.83×10^6 colony forming units of B067 Rz, R and S in

331   total volume of 100 µl in 0.5xN, 1x and 2xN Shieh media on Maxisorp plate. After a 44-

332   hour-incubation at RT, the emptied wells were rinsed twice with 200 µl of PBS. The biofilm

333   forming bacteria were stained with 125 µl of 0.1 % crystal violet solution for 10 minutes and

334   rinsed three times with 200 µl of PBS and the plate was dried at RT overnight. 125 µl of 96

335   % ethanol was added to solubilize the crystal violet. Finally, 100 µl of the solution was

336   transferred to a fresh microplate and absorbance was determined at the wavelength of 595 nm

337   with Multiscan FC spectrophotometer (Thermo Scientific).

338

Imaging bacterial cell movements

F. columnare B067 Rz cells were scratched from 0.5xN and 2xN Shieh agar plates and suspended in 0.5xN and 2xN Shieh liquid medium, respectively. The cell suspension was pipetted and imaged on 8-chambered Ibidi® Ibitreat µ-slide (Ibidi GmbH) covered with CID lid for µ-dishes (Ibidi GmbH). The bacterial cells were imaged with laser scanning confocal microscope Nikon AR1 using 488 nm Argon laser and CFI Apo VC 60x water immersion objective (numerical aperture 1.2).

In order to image the spreading behaviour of the different colony types, 3-5 µl of overnight culture of *F. columnare* B067 Rz, R or S was pipetted on 8-chambered Ibidi® Ibitreat µ-slide (Ibidi GmbH), between the bottom of the chamber and 0.5xN, 1x or 2xN Shieh agar layer. The bacteria were cultivated overnight at RT after which the motility on the edge of the spreading colony was imaged as described above. In order to make the slow bacterial movements detectable for human eye, the videos were sped up as follows: Supplementary Videos 1-8: 4×; Supplementary Video 9: 1800×.

Preparation of the samples for gene expression analysis

Several dilutions (with Shieh medium) were made from liquid bacterial cultures which were then spread on 0.5x and 2x Shieh agar plates in order to obtain plates with separate colonies and on which the colony types were recognizable. Plate cultures were incubated at RT for two days after which every plate was inspected to contain only the appropriate colony type. By diluting the bacterial cultures, close to round-shaped colonies were observed and their size was measured. A colony was considered as the area covered with bacterial cells, including the denser area in the middle of the colony (if present) and the more transparent area around it. Following the manufacturer's instructions, bacterial colonies were suspended in RNA Protect™ Bacteria Reagent (Qiagen) which protects RNA from degradation. Total RNA was extracted from Rz, R and S colonies grown on 0.5x and 2x Shieh agar plate cultures with RNeasy® Mini Kit (Qiagen). If there was any remaining genomic DNA, DNAse treatment with DNA-free™ (Ambion by Life Technologies) was carried out. RNA quality was verified by running the samples on Agilent RNA 6000 Nano chip (Agilent Technologies) for Agilent 2100 Bioanalyzer (Agilent Technologies) and determining the RNA integrity number (RIN) for each sample. Only qualified samples (RIN above 8.7) proceeded to cDNA synthesis immediately after RNA validation. RNA was reverse-transcribed into cDNA in triplicate

370   reactions with iScript™ cDNA Synthesis Kit (Bio-Rad) according to the instructions from the

371   manufacturer. cDNA reactions with the volume of 20 µl contained: 1x iScript reaction mix, 1

372   µl iScript™ reverse transcriptase, and 40 ng of template RNA. Replicate reactions were

373   pooled and used as template in qPCR.

374   RT-qPCR

375   Each 20 µl qPCR reaction, run in triplicates, contained 40 ng (*gapdh, glyA, gldG, gldH, gldL,*

376   *sprB*) or 80 ng (*sprA, sprE, sprF, sprT* and *porV*) of cDNA template, 0.5 µmol of both

377   forward and reverse primers (10 µmol) and 1X iQ™ SYBR Green Supermix (Bio-Rad) that

378   contained iTaq DNA polymerase (25 U/ml). qPCR reactions conditions were as follows: 95

379   ˚C for 3 min, followed by 40 cycles of 95 ˚C for 10 s, Tm ˚C for 20 s and 72 ˚C for 20 s,

380   (melting temperature (Tm) was chosen according to the primer pair (Table 1)). CFX96™

381   Real-Time System C1000™ and C1000™ Touch Thermal Cyclers (Bio-Rad) were used in

382   qPCR plate runs on 96-well Hard-Shell® PCR plates (Bio-Rad). On each plate, two interplate

383   calibrator samples in triplicates were run to normalize interplate variation.

384   Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to

385   design a primer pair for each target gene. Primers used in this study are presented in Table 1.

386   Specific binding of each primer pair was tested by checking the amplicon length on agarose

387   gel and with melt curve produced by CFX Manager™ Software v3.0. *glyA* and *gapdh* has

388   been qualified earlier as valid and stably expressed in each *F. columnare* colony type in

389   various nutrient conditions (23) and were thus used as reference genes to normalize the gene

390   expressions of gliding motility associated genes. M value (51), that indicates gene expression

391   stability, was measured for the current dataset for reference genes *glyA* and *gapdh* using CFX

392   Manager v3.0 (Bio-Rad Laboratories).

393   Relative quantities

394   For the following data prehandling, GenEx version 6.0 (MultiD Analyses) was utilized. Any

395   missing Cq value was replaced with the average Cq of its two qPCR replicates. IPC samples

396   run on each plate were used to minimize variation between different plate runs. Efficiency for

397   each primer pair was calculated from a standard curve (with CFX Manager version 3.0) and

398   Cq values were corrected with the efficiency within each gene. The averaged Cq values were

399   normalized with reference genes and transformed to relative gene expression with GenEx

400   version 6.0 (MultiD Analyses).

Statistical analyses

The effect of colony type and nutrient level on gliding motility gene expression was tested with ANOVA. Post hoc tests were bonferroni-corrected. Data for *sprA, sprE* and *gldG* were log-transformed to fulfil the assumption of normality and homoscedasticity. Statistical analyses were performed with IBM® SPSS® Statistics 22 (IBM Corporation), except for *gldL* (which could not be transformed to fulfil the assumption of ANOVA) which was analysed by ARTtool package in R (version 3.1.3.) (52). Therefore, for *gldL*, pairwise comparisons were not performed.

DNA sequencing of gliding motility genes in different colony types

Genomic DNA of *F. columnare* strain B067 colony types Rz, R and S was extracted from bacterial liquid cultures grown overnight in 1x Shieh medium at RT (115 rpm) using GeneJET Genomic DNA Purification Kit (Thermo Scientific). The genes related to gliding motility, T9SS or their regulation; *gldH*, *sprA*, *sprE*, *sprT*, *porV*, genomic regions spanning *sprCDBF, gldFG* and *gldKLMN* as well as partners of a putative two-component system, *porX* and *porY,* were first amplified with PCR using genomic DNA of B067 type Rz, R or S as a template. The 20 µl reactions were performed using Phusion Flash high-fidelity PCR master mix (ThermoFisher Scientific), with primer concentrations of 0.5 µM and template amount of 1-10 ng per reaction. The PCR protocol described by the manufacturer was followed, taking into account the differences in primer melting temperatures and PCR product sizes.

The organization of the genes studied in RT-qPCR assay within operons was predicted with DOOR2 (53, 54) according to the genome sequence of *Flavobacterium columnare* ATCC 49512. The genomic region upstream of the predicted operon was assumed to contain the appropriate promoter region. The upstream regions of operons *gldFG*, *gldKLMN* and *sprCDBF*, those operons comprising genes *gldH*, *sprA* or *porV* as well as the upstream region of gene *sprE* (that was predicted to be expressed alone) were sequenced in B067 Rz, R and S types.

Prior to sequencing the PCR products were purified using Qiagen's Qiaquick PCR purification kit. Primers for the sequencing reactions were designed in 500 bp intervals using VectorNTI v. 11.5.1 (Invitrogen), utilizing our shotgun sequencing results as template. A BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for sequencing the DNA fragments with Sanger sequencing technique with an automated

433   sequencing instrument, Applied Biosystems 3130*xl* Genetic Analyzer. The identity of each

434   base was determined with at least two good quality reads. Basecalling was done using

435   Sequence Analysis 6 (Applied Biosystems). Gene sequence assembly and the alignment of

436   homologous sequences of different colony types were performed with Geneious 8.1.5

437   (Biomatters Ltd). The assembled gene and regulative region sequences of Rz, R and S colony

438   types are found in GenBank (accession number in brackets); *gldFG* (MF278296), upstream

439   region of operon comprising *gldH* (MF278297), *gldH* (MF278298), *gldKLMN* (MF278299),

440   upstream region of operon comprising *sprA* (MF278305), *sprA* (MF278306), *sprCD*

441   (MF278307), *sprE* (MF278308), *sprF* (MF278309), *sprT* (MF278300), upstream region of

442   operon comprising *porV* (MF278301), *porV* (MF278302), *porX* (MF278303) and *porY*

443   (MF278304).

Protease activity and ECP production in different nutrient concentrations

445   To study the effect of nutrient level on proteolytic activity, B067 colony types Rz, R and S

446   were cultivated in 0.5xN and 2xN Shieh medium and 10 µl of bacterial culture (containing

447   1.4×10^6 CFUs +/- 4×10^4 S.E. on average) were spotted respectively on 0.5xN or 2xN

448   Shieh agar plates containing 1.5 % skim milk (Merck). Plates were incubated for 2 days at

449   RT after which the clear zone (indicating proteinase production) around the bacterial growth

450   was detected.

451   Extracellular product (ECP) samples were prepared as follows: eight ml from *F.*

452   *columnare* Rz, R and S liquid cultures were added to 100 ml of fresh 0.5xN and 2xN Shieh

453   media. The cultures were grown for 19 hours. 100 ml of dense bacterial culture was

454   centrifuged at 4 °C (4500 rpm, 15 minutes). The supernatant was first filtered through 0.45

455   µm Supor® membrane (Pall Corporation) and then concentrated with 10 K Amicon Ultra-15

456   Centrifugal Filter Units (Merck Millipore) at 4 °C to final volume of 2-3 ml. ECP samples

457   were divided in 500 µl aliquots and stored at -20 °C. Protein concentration of the ECP

458   samples was determined using Bradford method (55) against standard curve made with

459   known amounts of bovine serum albumin (BSA). 50 µg of each ECP sample (except 150 µg

460   of Rz grown in 2xN Shieh) was loaded on 14 % Tricine-SDS-PAGE gel. The gel was run for

461   24 hours at 90 V/30 mA and stained with Coomassie Brilliant Blue solution.

462 **AUTHOR CONTRIBUTIONS**

463 RP and L-RS designed the study. RP and VH conducted the laboratory experiments. RP, L-
464 RS and VH wrote the manuscript.

465 **FUNDING INFORMATION**

470 **ETHICS STATEMENTS**

471 This study does not involve human or animal subjects and therefore is not considered to
472 require ethical approval procedures.

473 **ACKNOWLEDGEMENTS**

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637

638 **FIGURES AND TABLES**

639 **Figure 1.** *F. columnare* **B067 colony types Rz, R and S growing on 0.5xN and 2xN Shieh**
640 **agar plates.** N refers to peptone and yeast extract concentrations which were either halved
641 (0.5xN) or doubled (2xN). See Figure 2 for the mean colony sizes.

642

643 **Figure 2. Growth characteristics of** *F. columnare* **B067 colony types Rz, R and S in**
644 **different nutrient levels.** Mean colony size (+/- S.E.) of colonies growing on 0.5x and 2x
645 Shieh agar plates **(A)**. Maximum optical density (A_{600}) (+/- S.E.) reached during a 65-hour-
646 liquid cultivation is presented as open circles **(B)**. Biofilm formation (A_{595}) (+/- S.E.) after 44
647 hours is presented as filled squares **(B)**.

648

649 **Figure 3. Organization of** *F. columnare* **B067 cells as microrhizoids on the edges of**
650 **biofilm.** Rz colony type grown under 1x Shieh agar layer **(A)** and S type under 0.5xN Shieh
651 agar **(B).** Scale bars 10 µm.

652

653 **Figure 4. Relative gene expressions of gliding motility and T9SS genes (+/- S.E.) in** *F.*
654 *columnare* **B067 colony morphologies Rz, R and S cultivated on 0.5x and 2x Shieh agar**
655 **plates.** The concentration of all Shieh components was either halved (0.5x) or doubled (2x)
656 compared to the normal Shieh medium. For detailed statistical analysis of the gene expression
657 results, see Tables 2 and 3.

658

659

660

661 **Table 1. Primer sequences and properties used in RT-qPCR study of *F. columnare*.**

Primer	Sequence	Amplicon length	Tm (°C)	Efficiency (%)	Reference
FC_gap1_fwd	ACCATCCCAAACAGGAGCCGC	98	56	105.7	Penttinen *et al*. 2016
FC_gap1_rev	CGTCTGCTGTAGGTACGCGCA				Penttinen *et al*. 2016
FC_glyA_fwd	CCAAACCCTTGGGGCTATACAACCC	98	60	102.8	Penttinen *et al*. 2016
FC_glyA_rev	AGAGGGCCTCCTTGATTACCTGGAA				Penttinen *et al*. 2016
FC_gldG_fwd	AGCAGAAGCAGTGATGCAGCA	125	58	100.95	This study
FC_gldG_rev	TGCCTTTGTAGGTAGCAATAGCCCA				This study
FC_gldH_fwd	CTTTGAAAACGGATGGCC	221	56	99.15	Klesius *et al*. 2010
FC_gldH_rev	CTTGCCCCATAAGACTTCC				Klesius *et al*. 2010
FC_gldL_fwd	GCAAGCGCTATGCTTATTGCTGGT	131	58	101.4	This study
FC_gldL_rev	GCAGTTGGTTGTCCCCCTGCT				This study
FC_sprA_fwd	GCAGAAAATGTTTGGCCCGT	162	60	99.95	This study
FC_sprA_rev	ACCGGCAGTTGCTCCATTAT				This study
FC_sprB_fwd	ACCAGCTGCTCCATGGTCAACTAC	157	60	100.1	This study
FC_sprB_rev	CGAAGGTGTCGTAGGGGCCG				This study
FC_sprE_fwd	AGCCGTGCAGAAGATAAAGC	151	60	100.8	This study
FC_sprE_rev	ACGCTTCTAATGCGGGTACAA				This study
FC_sprF_fwd	AGTCGTCAAATGGGGGCTAA	148	60	99.65	This study
FC_sprF_rev	TCACGCTTCCATCAAAGGTT				This study
FC_sprT_fwd	AACCAGGACTGCATTACGGA	144	60	101.1	This study
FC_sprT_rev	GCTTGATGTTACCTGTGCGTT				This study
FC_porV_fwd	GTGCCAACTCCTAAAACAGCC	152	60	96.85	This study
FC_porV_rev	AAACCTCCTGGAGCATCACC				This study

662

663

664 **Table 2. Statistical analysis of gliding motility and T9SS gene expression of *F*.**

665 *columnare* **B067 Rz, R and S colony types grown on 0.5x and 2x Shieh agar plates.**

Gene	Colony type			Nutrient			Colony type* nutrient interaction		
	F	df	p	F	df	p	F	df	p
sprB	0.225	2	0.800	6.583	1	0.016	0.185	2	0.832
gldG	10.148	2	<0.001	0.5223	1	0.476	1.289	2	0.291
gldH	6.093	2	0.006	0.229	1	0.636	3.918	2	0.032
gldL	7.118	2	0.003	19.605	1	<0.001	5.912	2	0.007
sprT	3.063	2	0.063	0.469	1	0.499	0.519	2	0.601
porV	2.268	2	0.122	0.654	1	0.426	0.493	2	0.616
sprA	0.807	2	0.456	21.363	1	<0.001	4.433	2	0.021
sprE	11.647	2	<0.001	0.448	1	0.509	3.382	2	0.048
sprF	1.681	2	0.204	7.312	1	0.012	1.072	2	0.356

666

667 **Table 3. Pairwise comparisons of gliding motility gene expression between *F. columnare***

668 **B067 Rz, R and S colony types and between growth conditions 0.5x and 2x Shieh agar**

669 **within a colony type.**

Gene	Pairwise comparisons of colony types			Nutrient 0.5x vs. 2x		
	Rz vs. R	*Rz vs. S*	*R vs. S*	*Rz*	*R*	*S*
sprB	NS	NS	NS	NS	NS	NS
gldG	<0.001	NS	0.004	NS	NS	NS
gldH	0.002	NS	0.009	NS	0.028	NS
gldL	0.0108	0.0087	NS	NP	NP	NP
sprT	0.030	NS	NS	NS	NS	NS
porV	NS	0.043	NS	NS	NS	NS
sprA	NS	NS	NS	<0.001	0.019	NS
sprE	<0.001	0.025	0.007	NS	NS	NS
sprF	NS	NS	NS	NS	0.041	NS

NS: statistically not significant result when confidence level of 0.95 was used; NP: Not performed

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