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**Author(s):** Penttinen, Reetta; Hoikkala, Ville; Sundberg, Lotta-Riina

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1           **Gliding motility and expression of motility-related genes in**  
2           **spreading and nonspreading colonies of *Flavobacterium***  
3           ***columnare***

4       Reetta Penttinen\*, Ville Hoikkala and Lotta-Riina Sundberg

5       University of Jyvaskyla, Centre of Excellence in Biological Interactions, Department of  
6       Biological and Environmental Science and Nanoscience Center, University of Jyvaskyla,  
7       Finland.

8       **\*Corresponding author:**

9       Reetta Penttinen  
10      University of Jyvaskyla  
11      Department of Biological and Environmental Science  
12      P.O. Box 35, FI-40014 University of Jyvaskyla, Finland.  
13      E-mail: reetta.k.penttinen@jyu.fi  
14      Tel. +358408053858

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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

19

20 **ABSTRACT**

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

40 **INTRODUCTION**

41 Gliding motility is a process of bacterial movement on surfaces in several bacterial species in  
42 the phylum Bacteroidetes (1). Instead of involvement of flagellae or pili, gliding motility is  
43 enabled by complex machinery which has been studied more closely in *Flavobacterium*  
44 *johsoniae* (for a review of *Flavobacterium* gliding motility, see (2)), a model system for  
45 gliding motility. Number of studies on flavobacterial gliding motility have led to  
46 identification of several genes involved in motility, including *gldA*, *gldB*, *gldD*, *gldF*, *gldG*,  
47 *gldH*, *gldI*, *gldJ*, *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprB*, *sprE*, *sprT* (3-15). Furthermore, a  
48 subset of these genes, *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE* and *sprT*, has been found to  
49 compose a protein translocation system, designated as type IX secretion system (T9SS) (1,  
50 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 *johsoniae* 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. johsoniae* (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 nonspreadng 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).

165 **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.

177 **DISCUSSION**

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

196 Each colony type was viable both at low and high nutrient conditions when they  
197 were cultivated in liquid medium. Even though each colony type succeeded better in high  
198 nutrient level (in liquid), they all expressed low colony spreading when they were cultivated  
199 under the same nutrient conditions on agar plate. This finding indicates that smaller colonies  
200 are not produced on high-nutrient-agar due to unfavourable growth conditions but rather as a  
201 result of reduced cell motility. In biofilm measurements, we found that Rz type was the most  
202 efficient biofilm producer in the low nutrient treatment. However, S type failed to produce  
203 biofilm, although this type increased colony spreading under low nutrient conditions. This  
204 indicates that spreading *per se* is not an indicator of biofilm formation. Biofilm formation is a  
205 process in which successful adhesion is required in order to attach to a surface (34). As the  
206 biofilm formation capacity of S type was comparable to control (growth medium without  
207 bacteria) it may be incapable of proper surface adhesion, possibly due to lack of functional  
208 cell surface adhesins. Indeed, the colonies of the S type are only moderately adherent (35)  
209 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

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

All the colony types showed proteolytic activity on high and low nutrient milk agar, indicating that colony spreading is not associated with secretion in this *F. columnare* strain. Previous studies have demonstrated that similarly to disruption of gliding motility, mutations in the gliding motility genes affect *F. johnsoniae* proteinase secretion (10). Therefore, our results indicate that the genes related to gliding motility and T9SS in the studied strain B067 are intact. Furthermore, no considerable differences in proteolytic activity were detected on low and high nutrient milk agar, even though in some cases low nutrient concentration has been shown to decrease protease activity (48). The ECP profiles of Rz, R and S types grown in low and high nutrient conditions did not differ remarkably from each other, except for a ~13 kDa protein band, detected in ECP, which was present only in Rz type. This protein has been connected to the virulent colony type also previously (22), but its role in pathogenicity is unknown. However, it should be noted that ECP profiles were isolated from bacteria grown in liquid media and therefore their ECP profiles may not fully correspond the profiles that may have been obtained from bacteria grown on an agar plate (and which would express gliding motility more vigorously).

To conclude, environmental nutrients are important regulators of *F. columnare* gliding motility and the expression of associated genes. Despite the mounting data of *Flavobacterium* gliding motility and T9SS, the knowledge of their regulatory pathways is limited, and how environmental cues contribute to the regulation of these signalling pathways in *F. columnare* remains to be determined. Therefore, it cannot be excluded that differentially spreading morphotypes of *F. columnare* are caused by some other factors that are yet to be identified. Understanding the differences of spreading and non-spreading morphotypes may help to disentangle factors related to gliding motility and virulence in *F. columnare*.

## 300 MATERIAL AND METHODS

### 301 Bacterial strains and growth conditions

In all of the experiments of this study, we used three different colony types (Rhizoid Rz, Rough R and Soft S) of *F. columnare* strain B067 (49). The bacterial strain was originally isolated from a trout that has been killed during columnaris disease outbreak at Finnish fish farm (49). The Rough colony type was obtained after exposing the original Rz isolate to bacteriophages (20). The Soft type appeared spontaneously during laboratory culture of the 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                   $1.18 \times 10^7$ - $1.28 \times 10^7$  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  $1.7 \times 10^6$ - $1.83 \times 10^6$  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

339 **Imaging bacterial cell movements**

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

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

353 **Preparation of the samples for gene expression analysis**

354 Several dilutions (with Shieh medium) were made from liquid bacterial cultures which were  
355 then spread on 0.5x and 2x Shieh agar plates in order to obtain plates with separate colonies  
356 and on which the colony types were recognizable. Plate cultures were incubated at RT for  
357 two days after which every plate was inspected to contain only the appropriate colony type.  
358 By diluting the bacterial cultures, close to round-shaped colonies were observed and their size  
359 was measured. A colony was considered as the area covered with bacterial cells, including  
360 the denser area in the middle of the colony (if present) and the more transparent area around  
361 it. Following the manufacturer's instructions, bacterial colonies were suspended in RNA  
362 Protect™ Bacteria Reagent (Qiagen) which protects RNA from degradation. Total RNA was  
363 extracted from Rz, R and S colonies grown on 0.5x and 2x Shieh agar plate cultures with  
364 RNeasy® Mini Kit (Qiagen). If there was any remaining genomic DNA, DNase treatment  
365 with DNA-free™ (Ambion by Life Technologies) was carried out. RNA quality was verified  
366 by running the samples on Agilent RNA 6000 Nano chip (Agilent Technologies) for Agilent  
367 2100 Bioanalyzer (Agilent Technologies) and determining the RNA integrity number (RIN)  
368 for each sample. Only qualified samples (RIN above 8.7) proceeded to cDNA synthesis  
369 immediately after RNA validation. RNA was reverse-transcribed into cDNA in triplicate

370 reactions with iScript<sup>TM</sup> 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<sup>TM</sup> 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<sup>TM</sup> 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<sup>TM</sup>  
381 Real-Time System C1000<sup>TM</sup> and C1000<sup>TM</sup> 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<sup>TM</sup> 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).

401 **Statistical analyses**

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

409 **DNA sequencing of gliding motility genes in different colony types**

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

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

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

433 sequencing instrument, Applied Biosystems 3130xl 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).

444 **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  $1.4 \times 10^6$  CFUs +/-  $4 \times 10^4$  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.

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**470 ETHICS STATEMENTS**

471 This study does not involve human or animal subjects and therefore is not considered to  
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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  $\mu$ m.

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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.

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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 <i>et al.</i> 2016
FC_gap1_rev	CGTCTGCTGTAGGTACCGCA				Penttinen <i>et al.</i> 2016
FC_glyA_fwd	CCAAACCCCTGGGGCTATACAACCC	98	60	102.8	Penttinen <i>et al.</i> 2016
FC_glyA_rev	AGAGGGCCTCCTTGATTACCTGGAA				Penttinen <i>et al.</i> 2016
FC_gldG_fwd	AGCAGAACGAGTGTGAGCA	125	58	100.95	This study
FC_gldG_rev	TGCCTTGTTGAGGTAGCAATAGCCCA				This study
FC_gldH_fwd	CTTGAAAACGGATGGCC	221	56	99.15	Klesius <i>et al.</i> 2010
FC_gldH_rev	CTTGCCCCATAAGACTTCC				Klesius <i>et al.</i> 2010
FC_gldL_fwd	GCAAGCGCTATGCTTATTGCTGGT	131	58	101.4	This study
FC_gldL_rev	GCAGTTGGTTGTCCCCCTGCT				This study
FC_sprA_fwd	GCAGAAAATGTTGGCCCGT	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	CGAAGGTGTCGTAGGGCCG				This study
FC_sprE_fwd	AGCCGTGCGAGATAAAGC	151	60	100.8	This study
FC_sprE_rev	ACGTTCTAATGCGGGTACAA				This study
FC_sprF_fwd	AGTCGTCAAATGGGGCTAA	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	GTGCCAACTCCTAAACAGCC	152	60	96.85	This study
FC_porV_rev	AAACCTCCTGGAGCATCACC				This study

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

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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
<i>sprB</i>	NS	NS	NS	NS	NS	NS
<i>gldG</i>	<0.001	NS	0.004	NS	NS	NS
<i>gldH</i>	0.002	NS	0.009	NS	0.028	NS
<i>gldL</i>	0.0108	0.0087	NS	NP	NP	NP
<i>sprT</i>	0.030	NS	NS	NS	NS	NS
<i>porV</i>	NS	0.043	NS	NS	NS	NS
<i>sprA</i>	NS	NS	NS	<0.001	0.019	NS
<i>sprE</i>	<0.001	0.025	0.007	NS	NS	NS
<i>sprF</i>	NS	NS	NS	NS	0.041	NS

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