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

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Running head: Gliding motility in *Flavobacterium columnare*

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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

the previously described secretion systems (1). T9SS has an important role in secretion of gliding motility adhesins, required for surface contact, but also for secretion of virulence factors (10, 16). For example, periodontal pathogen *Porphyromonas gingivalis* uses T9SS for secretion of its major virulence factors, gingipains and hemagglutinins (10, 16).

The exact role of each component in the gliding motility machinery of *F. johnsoniae* is not yet fully understood. GldB, GldD, GldH, GldI and GldJ are lipoproteins needed for gliding but their exact functions are not known (4, 5, 7-9). GldA, GldF and GldG form an ABC transporter but its role in gliding is still poorly known (3, 6). *sprB* is a motility adhesin needed for gliding and it is secreted via the T9SS (10). SprB encoding gene is located in operon *sprCDBF* where it is transcribed together with *sprC* and *sprD*, genes coding for proteins that support SprB function, and *sprF* which is needed for successful secretion of SprB (17). In addition, a recently identified secretion-related gene, *porV*, is needed for secretion of chitinase and adhesin RemA in *F. johnsoniae* (14). The mechanisms that control the assembly and activity of gliding motility machinery and T9SS are not known. In *P. gingivalis*, a two-component regulative system, consisting of PorX and PorY, regulates the expression of a subset of T9SS genes (10).

F. columnare is a fish pathogen belonging to phylum Bacteroidetes. *F. columnare* carries the majority of the orthologous genes (18) involved in *Flavobacterium* gliding motility and T9SS, which are used for virulence factor secretion and formation of spreading colonies (2, 10). Recently, a transcriptome-wide study in *F. columnare* strain ATCC 49512 demonstrated that genes associated with gliding motility and spreading are located in actively transcribed operons (19). *F. columnare* can form different colony morphotypes, spreading colony types Rz (rhizoid) and S (soft) and nonspreading type R (rough) (20, 21). Spreading colony morphology has been suggested to be essential for *F. columnare* virulence (20, 21), and indeed, only the spreading rhizoid Rz type is virulent in the fish host (20-22). Furthermore, changes in nutrient concentration in agar culture changes spreading of *F. columnare* colonies, especially in the virulent Rz type (20). Nutrient availability has also a significant impact on virulence in *F. columnare* as high nutrient level induces higher virulence in the bacteria (23, 24). However, the functionality of gliding motility and T9SS in different *F. columnare* morphotypes is not known, although *gldL*, *gldM*, *gldN*, and *gldH* have been suggested as putative virulence-associated factors in *F. columnare* (25, 26). Here, we explored gliding motility in *F. columnare* spreading (Rz, S) and nonspreading (R) morphotypes under conditions that were expected to induce (low-nutrient) or reduce (high-nutrient) spreading behaviour. Gliding motility and individual cell

movements were seen to be more active under low-nutrient conditions. We also performed a RT-qPCR (Reverse Transcription quantitative PCR) assay in order to measure the gene expression of T9SS or gliding motility -associated genes *gldG, gldH, gldL, sprA, sprB, sprE, sprF, sprT* and *porV*. Of these genes, *gldL, porV, sprA, sprE* and *sprT* are associated with the T9SS. Increased gene expression in response to low nutrient availability was detected in *sprA* and *sprF*. However, the spreading and nonspreading colony types had different expression profiles under different resource levels which could be an indication of divergent metabolic programs.

RESULTS

Nutrient availability regulates colony spreading in Rz and S morphotypes

The morphology of bacterial colonies originating from the same bacterial liquid culture and grown on 0.5xN or 2xN Shieh plates for 2 days was detected (Figure 1). Rz colonies grown on 0.5xN Shieh plate were spreading with increased mean colony size (3.95 cm, S.E. +/- 0.42) and production of root-like protrusions typical for Rz morphology (Figures 1 and 2A). Rz colonies grown on 2xN Shieh plates had smaller mean colony size (0.73 cm, S.E. +/- 0.04) and root-like structures, if seen, were only moderate (Figures 1 and 2A). Type S responded to changing nutrient availability comparably to Rz (mean colony size 2.4 cm, S.E. +/- 0.23 in 0.5xN and 0.75 cm, S.E. +/- 0.06 in 2xN). However, when grown at lower nutrient conditions (0.5xN Shieh), root-like structures were observed also in S type. These colonies were, nevertheless, distinguishable from Rz colonies by their non-adherent, opaque and moist colony appearance (Figure 1). R type did not remarkably alter the colony size in response to changing nutrient availability (mean colony size 0.7 cm, S.E. +/- 0.35 in 0.5xN and 0.5 cm, S.E. +/- 0.00 in 2xN) and root-like structures were seen under low nutrient conditions only occasionally (Figure 2A).

Growth and biofilm formation in varying nutrient concentrations

The viability of Rz, R and S colony types in 0.5xN and 2xN Shieh medium was measured as maximum optical density (OD_{max}) reached during a 65-hour-cultivation (Figure 2B). All the colony types reached the highest OD_{max} at higher nutrient level (2xN Shieh). The biofilm forming ability was remarkably higher under low nutrient condition (0.5xN Shieh) in the Rz

type compared to 2xN Shieh as well as R and S types, which were weaker biofilm producers in both nutrient levels (Figure 2B).

Imaging of bacterial movements

The movements of individual Rz cells grown on 0.5xN and 2xN Shieh agar plates was recorded with a confocal microscope. The movements of Rz cells were comparable with previously described gliding motility of *F. johnsoniae*: the cells glided over surface straightforwardly, occasionally attaching to the surface with one end of the cell, rotating and changing the moving direction (Supplementary Videos 1-2). The gliding speed was slower than that seen in *F. johnsoniae* which was used as reference (data not shown).

The movements of individual bacteria growing as a part of forming biofilm were recorded between agar layer and microslide chamber bottom. The colony types Rz and S formed a monolayer on the edges of spreading bacterial biofilm where the cells were organized side by side and glided along the adjacent cells (Figure 3; Supplementary Videos 3-4 and 7-8). The cells formed branching rhizoid-like structures, a few cells wide, (here referred to as microrhizoids) which involved both motile and non-motile cells. In both Rz and S types, more active gliding motility was seen under low nutrient concentration (0.5xN). Colony type R expressed only occasional movements regardless of the nutrient level and cellular organization as a spreading biofilm was not observed (Supplementary Videos 5-6). In order to visualize *F. columnare* colony formation at longer timescale, the growth of Rz colony type on 1x Shieh was recorded during an 8-hour recording (Supplementary Video 9). In the front of the biofilm, the bacteria were characteristically organized in microrhizoids which moved cooperatively towards the spreading direction of the biofilm and seemed also to serve as routes along which other cells were able to glide further and support biofilm expansion.

Sequence analysis and expression of gliding motility associated genes

Genes putatively involved in gliding motility were sequenced from Rz, R and S types of strain *F. columnare* B067. No genetic differences were detected between the colony types in the gene sequences of operons *gldFG* and *gldKLMN*, genes *gldH, sprA, sprC, sprD, sprE, sprF, sprT, porV, porX, porY* or in the predicted regulative regions upstream the genes *gldH, sprA, sprE, porV* or operons *gldFG, gldKLMN* and *sprCDBF*. Expression of genes *gldG, gldH, gldL, sprA, sprB, sprE, sprF, sprT* or *porV*, which are putatively involved in *F. columnare* gliding motility, was measured in B067 Rz, R and S colony types that had been

grown on 0.5x and 2x Shieh agar plates. Of these genes *gldL, sprA, sprE, sprT* and *porV* are associated with the T9SS. Gene expression results were normalized with reference genes *gapdh* and *glyA* which are stably expressed in the current dataset (M value 0.5834 for both genes with variance coefficient of 0.2114 for *gapdh* and 0.2007 for *glyA*). Relative expressions are presented in Figure 4 (for statistics, see Tables 2 and 3). Significant differences between colony types were observed in expression of genes *gldG, gldH, gldL* and *sprE* (Table 2, Figure 4, for pairwise comparisons see Table 3). Nutrient level had a significant effect on *gldL, sprA, sprB* and *sprF* expression. The pairwise comparisons revealed that *sprA* was expressed at significantly higher level in low-nutrient conditions in Rz and R types, and the same pattern was detected in *sprF* expression in R type (Table 3; Figure 4). However, significant interaction of colony type and nutrient was detected in *gldH, gldL, sprA* and *sprE* (Table 2), indicating that gene expression of colony types may differ between nutrient conditions. Indeed, direct associations between spreading behaviour and gliding motility gene expression were challenging to form as different colony types seemed to respond differently to the nutrient level with motility gene expression. Even though a significant effect of colony type was not observed in either *sprT* or *porV*, a significant difference between Rz and R was observed in *sprT* and between Rz and S in *porV* expression (Table 3).

Proteolytic activity and extracellular secretion in different nutrient concentrations

Colony types Rz, R and S were cultivated on 0.5xN and 2xN Shieh plates containing 1.5 % skim milk. Proteolytic activity was observed in each colony variant, seen as formation of a clear degradation zone peripheral to the bacterial growth, but no differences between the colony types were observed and the nutrient concentration did not affect to the proteolytic activity (Supplementary Figure S1). Effect of colony type and nutrient availability on contents of extracellularly secreted products (ECP) was analysed further. Generally, Rz, R and S grown in 0.5xN and 2xN liquid Shieh cultures shared a common overall ECP profile, with some moderate changes between individual protein bands between colony types (Supplementary Figure S2). However, a strong protein band, approximately 13 kD in molecular weight, was detected in Rz type grown in both 0.5xN and 2xN Shieh media. The 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.

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

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

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

We studied the gene expression of gliding motility or spreading -associated 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*.

MATERIAL AND METHODS

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).

Bacterial stocks were stored at -80 ˚C in 10 % FCS and 10 % glycerol and revived from the freezer in fresh modified Shieh medium according to Song *et al*. (50) which is referred to as Shieh medium in this study and used as a base of nutrient-modified media. In nutrient-modified media (0.5x or 2x Shieh), all the ingredients of the medium were either halved or doubled. In 0.5xN and 2xN Shieh media, only the concentration of peptone and yeast extract were either halved or doubled, respectively (for detailed compositions of the media, see (23)). After revival from the freezer, liquid bacterial cultures were cultivated at RT/26 ˚C, with agitation of 115/150 rpm for 24-48 h to obtain dense cultures. Cultures were refreshed with fresh 1x Shieh medium and cultivation was continued for 16-24 h. For plate cultures, dense liquid culture was streaked on Shieh agar plate which was incubated at RT for two days.

Colony morphology, growth and biofilm formation in different nutrient conditions

Liquid bacterial cultures of Rz, R and S grown in 1x Shieh were streaked on 0.5xN and 2xN Shieh plates. Plates were incubated for 2 days at RT after which the colony morphology was determined.

In order to evaluate the bacterial viability in different nutrient concentrations, 1.18×10^7-1.28×10^7 colony forming units of B067 Rz, R and S in total volume of 400 µl 0.5xN, 1x or 2xN Shieh (N=8) was cultivated on Honeycomb 2® microplate (Growth Curves Ltd) in Bioscreen C™ spectrophotometer (Growth Curves Ltd) at 26 ˚C. The absorbance (600nm) was measured every five minutes for 65 hours. The viability was estimated as the maximum absorbance recorded during the cultivation.

The biofilm formation capacity under various nutrient conditions was determined by cultivating 1.7×10^6−1.83×10^6 colony forming units of B067 Rz, R and S in total volume of 100 µl in 0.5xN, 1x and 2xN Shieh media on Maxisorp plate. After a 44-hour-incubation at RT, the emptied wells were rinsed twice with 200 µl of PBS. The biofilm forming bacteria were stained with 125 µl of 0.1 % crystal violet solution for 10 minutes and rinsed three times with 200 µl of PBS and the plate was dried at RT overnight. 125 µl of 96 % ethanol was added to solubilize the crystal violet. Finally, 100 µl of the solution was transferred to a fresh microplate and absorbance was determined at the wavelength of 595 nm with Multiscan FC spectrophotometer (Thermo Scientific).

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

reactions with iScript™ cDNA Synthesis Kit (Bio-Rad) according to the instructions from the manufacturer. cDNA reactions with the volume of 20 µl contained: 1x iScript reaction mix, 1 µl iScript™ reverse transcriptase, and 40 ng of template RNA. Replicate reactions were pooled and used as template in qPCR.

RT-qPCR

Each 20 µl qPCR reaction, run in triplicates, contained 40 ng (*gapdh, glyA, gldG, gldH, gldL, sprB*) or 80 ng (*sprA, sprE, sprF, sprT* and *porV*) of cDNA template, 0.5 µmol of both forward and reverse primers (10 µmol) and 1X iQ™ SYBR Green Supermix (Bio-Rad) that contained iTaq DNA polymerase (25 U/ml). qPCR reactions conditions were as follows: 95 ˚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, (melting temperature (Tm) was chosen according to the primer pair (Table 1)). CFX96™ Real-Time System C1000™ and C1000™ Touch Thermal Cyclers (Bio-Rad) were used in qPCR plate runs on 96-well Hard-Shell® PCR plates (Bio-Rad). On each plate, two interplate calibrator samples in triplicates were run to normalize interplate variation.

Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design a primer pair for each target gene. Primers used in this study are presented in Table 1. Specific binding of each primer pair was tested by checking the amplicon length on agarose gel and with melt curve produced by CFX Manager™ Software v3.0. *glyA* and *gapdh* has been qualified earlier as valid and stably expressed in each *F. columnare* colony type in various nutrient conditions (23) and were thus used as reference genes to normalize the gene expressions of gliding motility associated genes. M value (51), that indicates gene expression stability, was measured for the current dataset for reference genes *glyA* and *gapdh* using CFX Manager v3.0 (Bio-Rad Laboratories).

Relative quantities

For the following data prehandling, GenEx version 6.0 (MultiD Analyses) was utilized. Any missing Cq value was replaced with the average Cq of its two qPCR replicates. IPC samples run on each plate were used to minimize variation between different plate runs. Efficiency for each primer pair was calculated from a standard curve (with CFX Manager version 3.0) and Cq values were corrected with the efficiency within each gene. The averaged Cq values were normalized with reference genes and transformed to relative gene expression with GenEx 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

sequencing instrument, Applied Biosystems 3130*xl* Genetic Analyzer. The identity of each base was determined with at least two good quality reads. Basecalling was done using Sequence Analysis 6 (Applied Biosystems). Gene sequence assembly and the alignment of homologous sequences of different colony types were performed with Geneious 8.1.5 (Biomatters Ltd). The assembled gene and regulative region sequences of Rz, R and S colony types are found in GenBank (accession number in brackets); *gldFG* (MF278296), upstream region of operon comprising *gldH* (MF278297), *gldH* (MF278298), *gldKLMN* (MF278299), upstream region of operon comprising *sprA* (MF278305), *sprA* (MF278306), *sprCD* (MF278307), *sprE* (MF278308), *sprF* (MF278309), *sprT* (MF278300), upstream region of operon comprising *porV* (MF278301), *porV* (MF278302), *porX* (MF278303) and *porY* (MF278304).

Protease activity and ECP production in different nutrient concentrations

To study the effect of nutrient level on proteolytic activity, B067 colony types Rz, R and S were cultivated in 0.5xN and 2xN Shieh medium and 10 µl of bacterial culture (containing 1.4×10^6 CFUs +/- 4×10^4 S.E. on average) were spotted respectively on 0.5xN or 2xN Shieh agar plates containing 1.5 % skim milk (Merck). Plates were incubated for 2 days at RT after which the clear zone (indicating proteinase production) around the bacterial growth was detected.

Extracellular product (ECP) samples were prepared as follows: eight ml from *F. columnare* Rz, R and S liquid cultures were added to 100 ml of fresh 0.5xN and 2xN Shieh media. The cultures were grown for 19 hours. 100 ml of dense bacterial culture was centrifuged at 4 °C (4500 rpm, 15 minutes). The supernatant was first filtered through 0.45 µm Supor® membrane (Pall Corporation) and then concentrated with 10 K Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) at 4 °C to final volume of 2-3 ml. ECP samples were divided in 500 µl aliquots and stored at -20 °C. Protein concentration of the ECP samples was determined using Bradford method (55) against standard curve made with known amounts of bovine serum albumin (BSA). 50 µg of each ECP sample (except 150 µg of Rz grown in 2xN Shieh) was loaded on 14 % Tricine-SDS-PAGE gel. The gel was run for 24 hours at 90 V/30 mA and stained with Coomassie Brilliant Blue solution.

AUTHOR CONTRIBUTIONS

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

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ETHICS STATEMENTS

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

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FIGURES AND TABLES

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

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

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

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

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

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663

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

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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