Different colony morphologies enlighten the way to the tracks of *Flavobacterium columnare* virulence factors

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Abstract

Flavobacterium columnare is a fish pathogen that causes columnaris disease in salmonid fish. Severe symptoms (skin lesions, fin erosion and gill necrosis) caused by the infection are often lethal. During the last decades columnaris disease has become a serious problem for fish farming industry not only at fish farms in Finland, but outbreaks have been reported around the world [1]. *F. columnare* infects especially young salmonids and as an effective transmitter it can cause up to 100 % mortality as it benefits from the high population densities in which fish are produced. Despite the importance of columnaris disease, the virulence mechanisms of the pathogen are poorly understood.

When grown under laboratory conditions on a solid surface, like on an agar plate, *F. columnare* can form three different colony types: rhizoid, rough and soft. Our previous studies have indicated that only the rhizoid morphotype is virulent in fish and able to move on surfaces by gliding. Rough and soft morphotypes of *F. columnare* are non-virulent but differ from each other in their adherence and gliding motility. Rough type is very adherent and non-gliding whereas soft type glides but does not attach on surfaces [2]. This suggests that virulence of *F. columnare* is a combination of at least three factors: rhizoid colony morphology, gliding motility and adherence. Indeed, the rhizoid type is isolated from disease outbreaks but during laboratory culture other colony morphotypes appear on agar plates. Switch in the colony morphotype can be reversible and it occurs spontaneously or it can be triggered by a stress factor such as bacteriophage [3] or lack of nutrients [4].

Flavobacterial gliding motility has been characterized in *F. johnsoniae.* Gliding motility machinery is a protein complex composed of ~20 different proteins including proteins encoded by *gld* and *spr* genes [5-9]. SprB is part of gliding motility machinery and serves as an adhesive protein that is propelled along the cell surface [8]. SprT is involved in secretion system which serves in translocation of proteins such as SprB or chitinase [9]. Recently it was found, that the flavobacterial gliding motility machinery is orthologous to the PorSS (Por secretion system) that is used as a secretion system of virulence factors in another *Bacteroidetes* bacterium *Porphyromonas gingivalis* [9]. Because the activity of the connective tissue degrading enzyme, chondroitin AC lyase, has been reported to be related to virulence of *F. columnare* [10] and previously found correlation between motility and virulence, we decided to study the expression of the possible virulence-related factors of *F. columnare*. Furthermore, the bacterium's ability to

switch between colony types led us to propose that virulence is due to exquisitely regulated gene expression. Gene expression was studied using RT-qPCR and expression patterns were compared between all three colony types (rhizoid Rz, rough R and soft S) of the strain B67 cultivated in different nutrition concentrations.

Bacteria were cultivated in Shieh medium [11] and on Shieh plates with different nutrient conditions (2X, 1X and 0.5X Shieh). The colony diameter of the bacterial colonies in different nutrient concentrations was measured and RNA was isolated from fresh samples for the gene expression studies. Expressions of chondroitin AC lyase and genes involved in PorSS/gliding motility (*gldG, gldH, gldL, sprB* and *sprT*) were measured using RT-qPCR (reverse transcription quantitative PCR). Two reference genes *gapdh* and *glyA* (glyceraldehyde-3-phosphate dehydrogenase and serine hydroxymethyltransferase, respectively) were used to normalize the results and suitability as reference genes was confirmed (M value= 0.6111 for both).

Change in colony diameter in response to nutrient concentration indicated the bacterium's ability for gliding motility. In virulent rhizoid (Rz) and non-virulent soft (S), colony diameter increased significantly towards decreasing nutrient concentration ($P<0.001$) whereas rough (R) had no ability to adapt to lack of nutrients (Fig. 1). The result is in accordance with our previous studies and suggests that low nutrient level induces motility because bacteria are forced to search for food elsewhere.

In liquid culture, no clear differences between colony types in expression of gliding motility genes (*gldG, gldH, gldL, sprB* and *sprT*) were found, but the expression of chondroitinase was significantly higher in the rhizoid morphotype than in other morphotypes (Fig. 2). The expression of motility genes may not be important during the planktonic growth of *F. columnare* because the gliding motility machinery is used for gliding over surfaces.

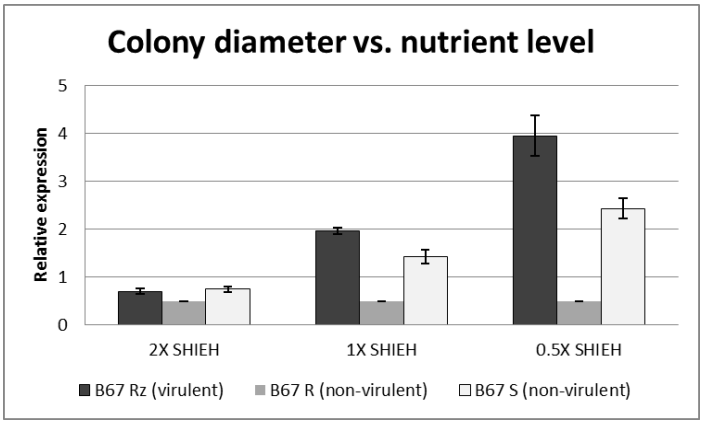

Figure 1. Colony diameters of *F. columnare* colony types in different nutrient conditions.

On agar plate cultures, the expressions of the *gld* and *spr* genes were not significantly affected by colony morphotype or nutrient concentration although the colony diameter of both Rz and S morphotype bacteria were significantly influenced by nutrient conditions ($P<0.001$; Fig. 1).

These results suggest that both gliding motility and adherence on surfaces are complex in *F. columnare*. It is also possible that *gld* and *spr* genes are expressed but the proteins are not functional or not located on the cell envelope but they accumulate inside bacterial cell. It has already been reported that mutation in one gene of the gliding motility machinery may lead to the disruption of the whole protein complex [12].

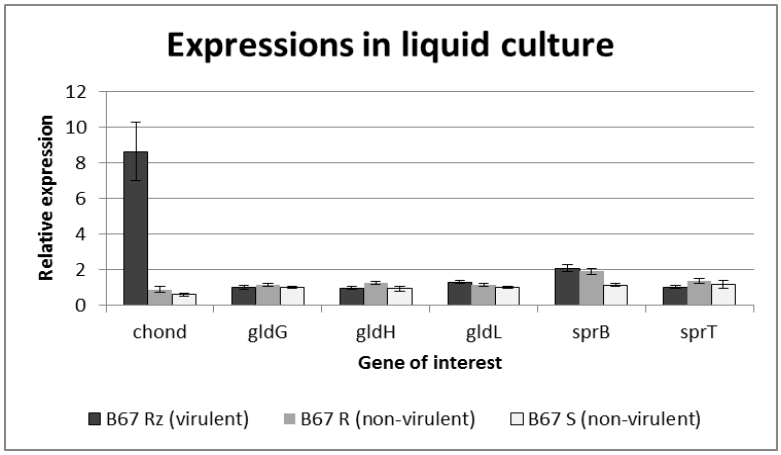

Figure 2. Gene expressions of possible virulence-related genes of *F. columnare* colony types in liquid culture.

The expression of chondroitinase was higher in the virulent rhizoid than non-virulent morphotypes also in agar cultures, suggesting that chondroitinase is indeed expressed as a virulence factor in *F. columnare* (Figs. 2 and 3). In addition, expression of chondroitinase correlated positively with nutrient level in all colony types. It is possible that the chondroitinase production is induced by high nutrient concentration that resembles the presence of a fish host, whereas in low nutrient levels (like in water) it is not necessary to express virulence factors.

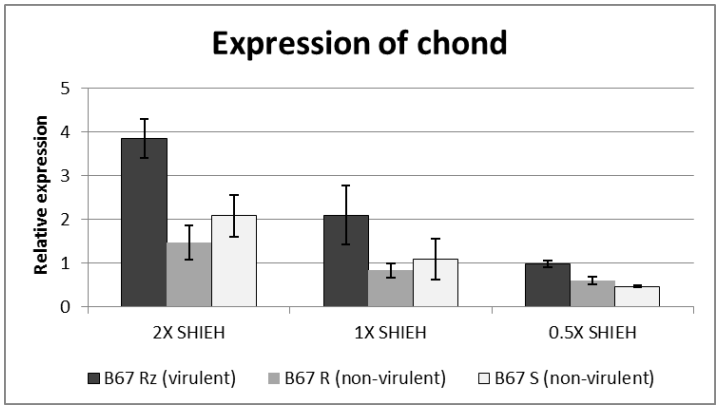

Figure 3. Expression of chondroitinase in plate cultures with different nutrient conditions.

To conclude, studying colony morphotypes with different virulence capacities can reveal us virulence factors and the environmental cues that may regulate the expression of virulence in *F. columnare*. However, more studies are needed to confirm the genetic background of the PorSS/gliding motility system and virulence in *F. columnare*.

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