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Flavobacterium columnare colony types: Connection to adhesion and virulence?

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ABSTRACT

Four different colony morphologies were produced by *Flavobacterium columnare* strains on Shieh agar plate cultures: rhizoid and flat (type 1), non-rhizoid and hard (type 2), round and soft (type 3), and irregularly shaped and soft (type 4). Colonies produced on AO agar differed from these to some extent. The colony types formed on Shieh agar were studied according to molecular characteristics [Amplified Fragment Length Polymorphism (AFLP), Automated Ribosomal Intergenic Spacer Analysis (ARISA), and whole cell protein SDS-PAGE profiles], virulence on rainbow trout fingerlings, and adhesion on polystyrene and fish gills. There were no molecular differences between colony types within one strain. Type 2 was the most adherent on polystyrene, but type 1 was the most virulent. Adhesion of *F. columnare* strains used in this study was not connected to virulence. From fish infected with colony type 1, three colony types (types 1, 2 and 4) were isolated. Contrary to previous studies, our results suggest that strong adhesion capacity may not be the main virulence factor of *F. columnare*. Colony morphology change might be caused by phase variation, and different colony types isolated from infected fish may indicate different roles of the colony morphologies in the infection process of columnaris disease.

1. Introduction

Flavobacterium columnare, the causative agent of columnaris disease, is a freshwater bacterium that can be isolated from natural waters [1,2]. Nowadays, the bacterium is considered as one of the most harmful bacterial fish pathogen at freshwater fish farms worldwide: columnaris outbreaks cause remarkable financial and material losses yearly for the fish farming industry (see Ref. [3]). Development of improved cultivation methods (*e.g.* Refs. [4,5]) has enabled a routine isolation of *F. columnare*, facilitating the diagnostics and studying of this pathogen in laboratory conditions.

Despite its pathogenicity, virulence mechanisms of *F. columnare* are largely unknown. It is known, that different genetic groups [6–8] express different degrees of virulence. It has also been shown that the activity of connective tissue degrading enzyme, chondroitin AC lyase [7], and capacity to adhere on gill tissue [9] are related to virulence of *F. columnare*. Growth characteristics in different culture conditions [10], production of extracellular proteases [11,12] and outer membrane protease genes [13] have been studied, but clear correlation to virulence has not been observed. On the other hand, differences in lipopolysaccharide (LPS) and protein profiles between virulent and avirulent *F. columnare* strains have been detected [14]. In some pathogenic bacteria, cell surface components, such as LPS and capsular material, function as virulence factors (*e.g.* Refs. [15–17]).

Change in cell surface components often leads to change in colony morphology of a bacterium (see *e.g.* Refs. [16–19]). In some human and animal (also fish) pathogens, such as tubercle bacilli [20], *Vibrio vulnificus* [21], and *Mycobacterium avium* [22], different colony morphologies produced by one strain can exhibit difference in virulence. Previously, difference in colony morphology has been detected between *F. columnare* strains [23]. Also non-rhizoid as well as soft and non-adherent colonies have been noticed to appear among rhizoid colonies after subcultivation [24,25]. It has been shown, that spreading or rhizoid colony formation is an indication of gliding motility of *Flavobacterium johnsoniae* (previously *Cytophaga johnsonae*) [26] and *Flavobacterium psychrophilum* (previously *Cytophaga psychrophila*) [27]. Therefore, it is possible, that the loss of gliding motility appears as non-rhizoid colony morphology also in *F. columnare*. Different colony morphologies have also been found in *Flavobacterium succinicans* (previously *Cytophaga succinicans*), a relative to *F. columnare* [28]. However, the pathogenesis of colony morphology variants has not been studied in flavobacteria.

We found that four different colony morphologies are formed among *F. columnare* strains on Shieh agar plates in laboratory cultivations, and one strain can form one or two morphology variants. Because of severity of repeated infections at fish farms and lack of knowledge of virulence mechanisms in *F. columnare*, we consider it important to study further these colony types. This is necessary both for developing exact diagnostic tools for columnaris infections and also to find out whether there is a connection

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between virulence and colony morphology. This is why molecular characteristics and adhesion properties as well as fish mortality caused by different colony morphologies were studied.

2. Results

Four different colony types (Fig. 1) were formed on Shieh agar plates in laboratory conditions by the eight *F. columnare* strains tested (Table 1). Colony type 1 was rhizoid and flat with yellow centre. Colony type 2 was hard, more orange in color, non-rhizoid or only slightly rhizoid, and had irregular edges and convex growth form. Colony type 3 had round edges, and smooth, yellowish appearance. Type 4 colonies were white or light yellow, smooth and spreading on the agar with irregular shape. Originally, *F. columnare* strains formed colony types 1–4 (Table 1), each strain producing only one colony type. In further plate cultivations other colony types started to form among original types in a following manner: type 1 → type 2, type 2 → type 4, and type 3 → type 4, meaning that among type 1 colonies, type 2 colonies started to appear, etc. These other colony types all formed in the cultures of same age and, once formed, remained the same from generation to generation in laboratory cultivations. There was no colony type change in the opposite direction meaning, that no type 3, 2 or 1 colonies were formed among type 4 colonies, no type 2 or 1 colonies among type 3 colonies, and no type 1 colonies among type 2 colonies. Colony type did not change in broth cultivations. In older cultures (more than 3 days after plate cultivation), however, on the edges of some types 2 and 3 colonies growth resembling type 4 started to appear. There were no contaminations of other bacteria or between *F. columnare* strains in the cultures. Colony type 4 existed only in the genetic groups with low or intermediate virulence, whereas colony type 3 existed only in high virulence strain. On AO agar, formation of colony morphologies differed from that on Shieh agar to some extent: The growth form of bacteria was more spreading on AO agar than on Shieh agar. Moreover, the older cultures on AO agar started to get transparent appearance, which did not occur on Shieh agar. The growth form of all bacteria of all genetic groups resembled colony type 4 formed on Shieh agar, but had more spreading colonies with slightly rhizoid edges. The exceptions were genetic groups B and F, which formed types 3 and 4 (formed on Shieh agar) resembling colonies also on AO agar, respectively. Among rhizoid type 4 colonies, also types 1 and 2 (formed on Shieh agar) resembling colonies were formed by bacteria of genetic groups D, E and G. None of the colony types 1–4 formed on AO agar had exactly the same appearance as the corresponding colony types 1–4 formed on Shieh agar, but could be categorized into these groups. However, because of the spreading growth form of bacteria on AO agar, the different colony types grew rather stuck on each other than as separate colonies. On AO agar, the colony types 1, 2 and 4 formed originally on Shieh agar grew as rhizoid type 4 colonies. Types 1 and 2 (formed on Shieh agar) resembling colonies were also detected among D2 (colony type 2 formed by strain D), E1, E2 and G2.

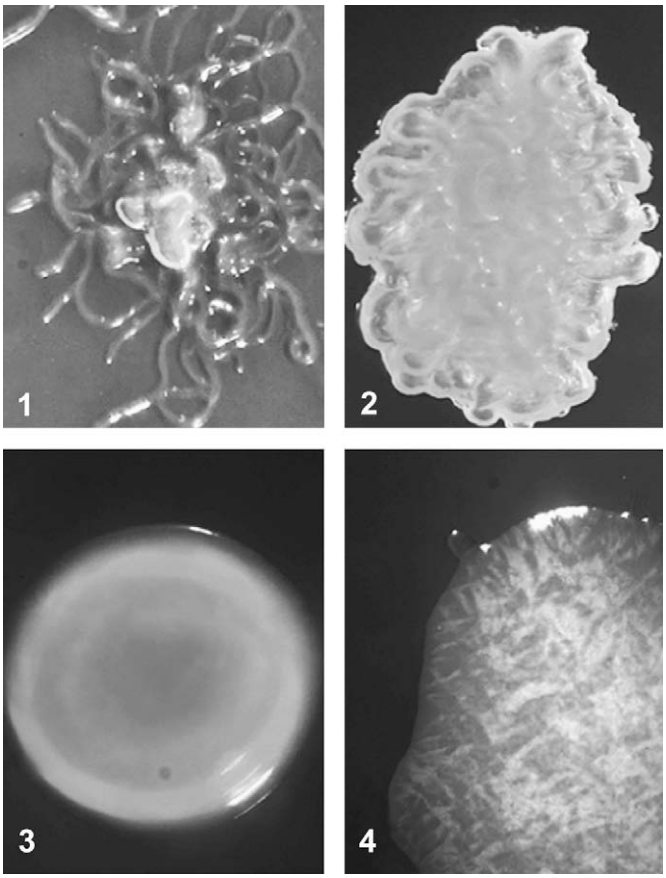

Fig. 1. Representatives of different colony morphologies (1 = colony type 1, 2 = type 2, 3 = type 3, 4 = type 4) formed by *Flavobacterium columnare* (see also Table 1) on Shieh agar plate cultivation in laboratory. Type 1 is rhizoid and flat with yellow centre. Type 2 is hard, more orange in color, non-rhizoid or only slightly rhizoid, and has irregular edges and convex growth form. Type 3 has round edges, and smooth, yellowish appearance. Type 4 colonies are white or light yellow, smooth and spreading on the agar with irregular shape.

There were no differences in ARISA [10], AFLP or whole cell protein SDS-PAGE (Fig. 2) profiles between different colony types formed by one *F. columnare* strain. However, there were differences in adhesion capacities on polystyrene (Kruskal–Wallis test, overall comparison between strains A–H forming colony types 1–4: $\chi^2 = 66{,}947$, df = 12, $P < 0.001$) (Fig. 3a): D2 and D4, E1 and E2, G2 and G4, as well as H2 and H4 differed significantly from each other. Similarly, on polystyrene, there was a significant difference between adhesion capacities of colony types (one-way ANOVA: $F = 36.679$, df = 4, $P < 0.001$) (Fig. 3b); colony type 2 was more adherent than other colony types, but also the adhesion of colony type 1 differed significantly from the other colony types. The growth rate of bacteria did not affect the adhesion capacities. On agar, colony type 2 was the most adherent followed by types 1, 3 and 4. This result was achieved by the experience when handling the colonies with inoculation loop. Adhesion capacities on gill tissue differed significantly between colony types among the strains (one-way ANOVA: $F = 3{,}306$, df = 6, $P = 0.031$) (Fig. 3c). G4 was the most adherent and differed significantly from all the other colony types tested, except from E2. A significant difference between adhesion capacities of E2 and H4 was also detected.

In challenge experiment, there was a significant difference between mortalities caused by the colony types (Kaplan–Meier

Table 1
Colony morphology types formed on Shieh agar in laboratory conditions by genetically grouped *Flavobacterium columnare* strains (see Refs. [10] and [7] for more details on the strains).

Genetic group	Virulence of the strain	Original colony type	Colony type formed in subcultivations
A	Low	1	
B	High	3	
C	High	1	
D	Low	2	4
E	High	1[a]	2[a]
F	Low	4	
G	Intermediate	2[a]	4[a]
H	Low	2[a]	4[a]

[a] Colony type was used in the virulence experiment using rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings.

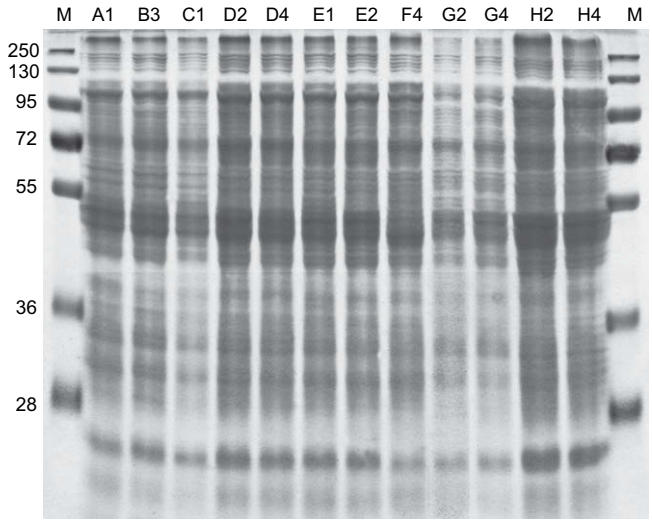

Fig. 2. Whole cell protein SDS-PAGE profiles of *Flavobacterium columnare* strains A–H forming colony types 1–4 (see Table 1). In the first and the last lane are the molecular mass markers (kDa).

survival analysis: $\chi^2 = 187.483$, df = 6, $P < 0.001$) (Fig. 4). Interestingly, the most polystyrene-adherent colony type 2 did not produce the highest mortality. The cumulative mortality caused by E1 was the highest, 60%, and statistically different from mortalities caused by the other colony types tested (E2, G2, G4, H2 and H4) (Table 2). The symptoms of columnaris disease (skin and/or gill lesions) were detectable in all fish infected with E1, but present approximately only in half of the fish infected with the other colony types. *F. columnare* could be isolated only from fish infected with colony type E1. Interestingly, from primary cultivations made from these fish, several colony types per one fish could be isolated: in addition to colony type 1, the isolated bacteria were also of colony types 2 and 4.

3. Discussion

This is the first time when spontaneously formed colony morphologies among *F. columnare* strains are studied to find connection between colony types, adherence and virulence. Colony types among each *F. columnare* strain showed no genomic differences in ARISA, AFLP, or whole cell protein SDS-PAGE profiles. The four colony types formed on Shieh agar by *F. columnare* strains differed significantly in their adhesion capacities on polystyrene and agar; type 2 was the most adherent followed by types 1, 3 and 4. Virulence, however, was not related to adherence capacity: strongly polystyrene- and agar-adherent type 2 bacteria (E2, G2 and H2) were not more virulent than rhizoid type 1 (E1) or poorly adherent type 4 (G4 and H4) bacteria. *F. columnare* could be isolated only from fish dead in E1 infections, which may be due to difficulties in isolation of *F. columnare* at preclinical stages of infection, or possible presence of competitive bacteria [29]. Our results suggest that strong adhesion is not essential for virulence of *F. columnare*. In addition, adhesion to gill tissue was not related either to virulence or adherence on polystyrene, indicating that same molecules are not used to attach on different surfaces, and that virulence mechanisms other than adhesion may be important in pathogenesis of *F. columnare*. Formation of different colony types occurred also when *F. columnare* was grown on AO agar, but colonies were not exactly similar to and did not grow as clear separate colonies as the colonies formed on Shieh agar. Differential colony formation on Shieh and AO agar may have been caused by different nutrition composition between these two media. Shieh medium is

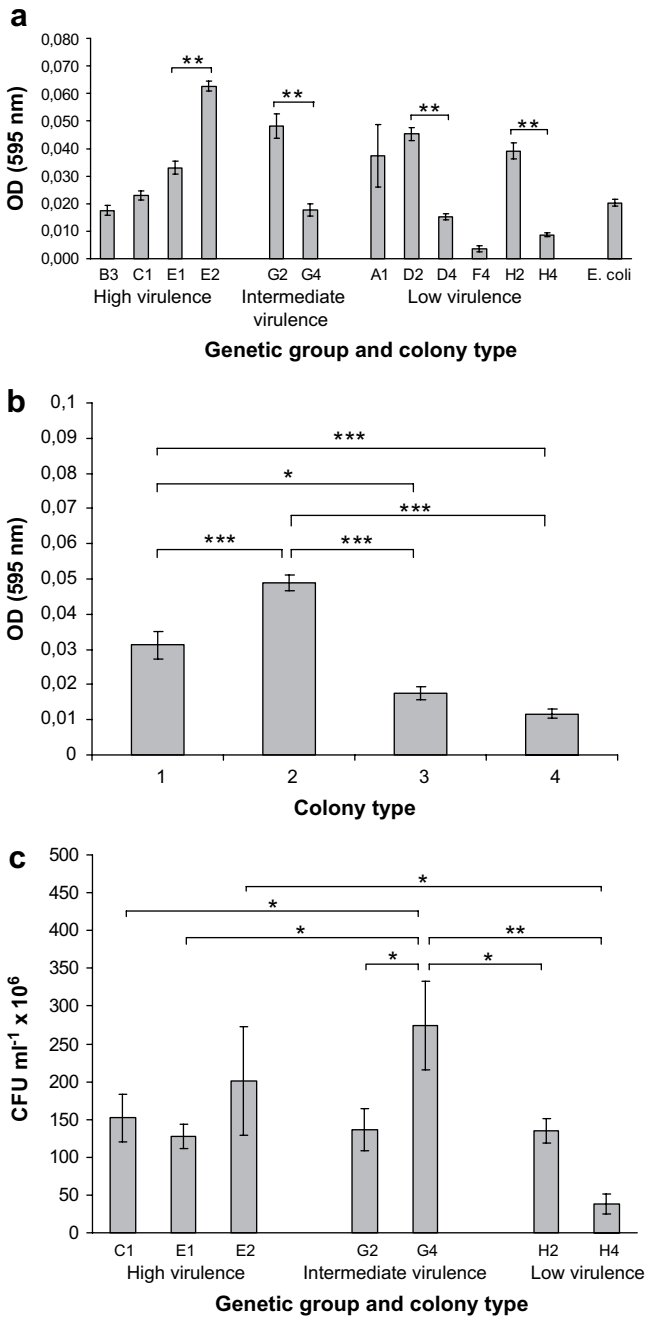

Fig. 3. (a). Adherence (mean ± SE) on polystyrene of *Flavobacterium columnare* strains A–H forming colony types 1–4 (see Table 1), and *Escherichia coli*. Statistical differences (Kruskal–Wallis test, pairwise comparisons) between different colony types formed by one strain: $^{*} = P < 0.05$, $^{**} = P < 0.01$, $^{***} = P < 0.001$. (b). Adherence (mean ± SE) on polystyrene of *Flavobacterium columnare* strains (A–H) representing the same colony morphology type (1–4) (see Table 1). Statistical differences (one-way ANOVA, pairwise comparisons, Post Hoc LSD test) between different colony types: $^{*} = P < 0.05$, $^{**} = P < 0.01$, $^{***} = P < 0.001$. (c). Adherence (mean ± SE) on gill tissue (rainbow trout (*Oncorhynchus mykiss*, Walbaum) of *Flavobacterium columnare* strains C, E, G and H forming colony types 1–4 (see Table 1). Statistical differences (one-way ANOVA, pairwise comparisons, Post Hoc LSD test) between the strains: $^{*} = P < 0.05$, $^{**} = P < 0.01$, $^{***} = P < 0.001$).

specific for *F. columnare* [5] and might thus allow broader variation of colonial morphology than AO medium.

Change in colony morphology is not a rare phenomenon; it occurs in many bacterial species pathogenic to humans and animals. For example, colony morphology variation between translucent and opaque colony types is observed both in *Vibrio parahaemolyticus* [30] and *V. vulnificus* [21], whereas *M. avium* [22]

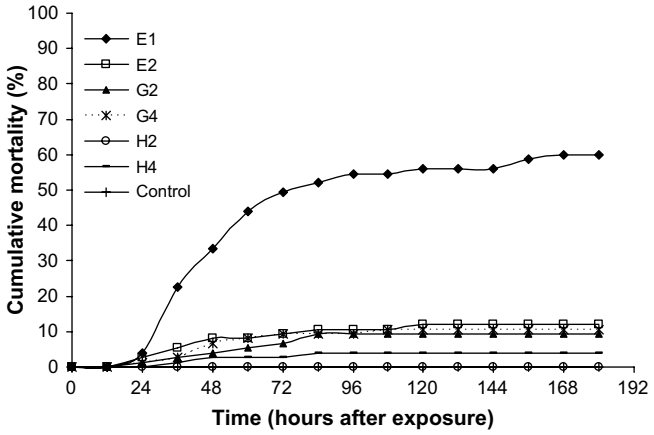

Fig. 4. Average cumulative mortality of rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings challenged with equal number of bacteria representing different morphology types (types 1, 2 and 4, see Table 1) formed by *Flavobacterium columnare* genetic groups E (high virulence), G (intermediate virulence) and H (low virulence).

represents smooth and rough colony types. Colony morphology change is sometimes caused by a phenomenon known as phase variation (reviewed in Refs. [31,32]). Phase variation is often thought to be a random event occurring in a bacterial population as a consequence of heritable genomic changes which can be seen as subpopulations of different phenotypes [33]. Most of the changes are caused by more or less radical genomic rearrangements, like DNA inversions or recombinational deletions, but some minor DNA modifications, like differential DNA methylation, are also known to lead to phase variation. However, growing amount of evidence suggests that phase variation is not always a random event, but a response to an environmental change and caused by change in gene expression [31]. Phase variation allows bacteria to adapt the surrounding environment and is caused by a reversible on/off switch in the expression of one or more genes [32]. Switch can be triggered by intra- or extracellular activators or inhibitors, such as temperature [34], or signaling molecules received from the environment [32]. These molecules can be related to *e.g.* bacterial quorum sensing. Quorum sensing is a chemical signaling system which is used by bacterial populations to control variety of functions via regulation of gene expression [35]. Besides chemicals produced by bacteria, molecules received from a host can also affect behavior of bacterial populations [36,37].

Production of non-typical colony morphologies after subcultivation has been previously detected also in *F. columnare* [25]. In another study, non-adherent *F. columnare* colony variant was obtained by β-lactam selection [24]. In these studies, no genetic methods were used to find out whether different colony formation is caused by changes at the DNA level. Studying the properties of different colony types can provide information about virulence and transmission strategies of *F. columnare*. It is possible that change in colony type of *F. columnare* is caused by phase variation affecting the expression or modification of some bacterial surface molecule. However, because no genomic changes were detectable by ARISA, AFLP or whole cell protein SDS-PAGE, the changes may be only minor or may take place at the gene expression level. In human pathogens *E. coli* and *Salmonella enterica* serotype Typhimurium phase variation occurs as a consequence of different DNA methylation, which does not lead to change in DNA sequence [32]. In *V. parahaemolyticus* genetic alterations in cell type controlling transcription factor coding *opaR* gene may lead to formation of different colony types [30]. Although phase variation is usually considered as a reversible phenomenon, also irreversible changes in bacterium's phenotype can be caused. For example, irreversible phase variation occurs in a human pathogen *V. vulnificus* [18]. Also in the present study, the change in colony type was found to be irreversible. Possible phase variation occurring in *F. columnare* may be a consequence of a change in gene expression and a response to a change in growth environment. It is known, for example, that phase variation occurs during starvation in *V. vulnificus* [38] and *Neisseria gonorrhoeae* [39]. The effect of change in nutritional conditions of the growth environment on *F. columnare* phenotype could be seen also in this study as difference between colony morphology formation on Shieh and AO agars.

In addition to changes in growth environment, also quorum sensing has a central role in phase variation and biofilm development in some bacteria, for example in *V. parahaemolyticus* [19]. Biofilm production properties are needed in the beginning of *V. cholerae* infection, but suppressed later by quorum sensing [40]. Quorum sensing also controls virulence gene expression in *V. cholerae* [41]. Quorum sensing has not been studied in *F. columnare*, and in *F. psychrophilum* no evidence of quorum sensing was detected, when acylated homoserine lactone (AHL)-based quorum sensing signaling system was studied [42]. However, among AHL-based signaling also several other quorum sensing systems are known and the possible existence of quorum sensing system in *F. columnare* cannot be excluded. Isolation of colony types 1, 2 and 4 from fish infected with E1 indicates that different colony types may have different roles in the infection cycle, but whether they are formed by phase variation as a response to nutritional factors or quorum sensing can only be speculated and needs more studies. However, it is known that, especially in an animal host, the environment can drive the selection against a particular phenotype of a bacterium, which can result in a different phenotype for cells cultured in the laboratory and those isolated from the host [32]. Indeed, phase variation is considered as one mechanism of pathogenic bacteria to avoid immune defense of a host [31].

Our results show, that there is no clear relation between adhesion capacity and virulence of *F. columnare* strains tested in this study. We have previously tested adhesion capacity of *F. columnare* also on crude mucus-coated slides [7], and similarly no correlation to virulence was detected. These results contradict the previous study by Bader et al. [24]. In their immersion challenge experiment the smooth colony forming, less virulent, *F. columnare* mutant was significantly less adherent both on skin and gill tissue of channel catfish (*Ictalurus punctatus*) than wild type strain, which formed hard colonies with finger-like projections and which may have resembled our colony type 1. However, in their study, Bader et al. did not detect colony morphologies which would have been adherent and hard with no finger-like projections, *i.e.* similar to our colony type 2. This may explain the difference in results compared to our study: the virulent strain in that study resembling our high virulence colony type 1 was also the most adherent and Bader et al. did not detect highly adherent type 2 colonies, which produced less mortality in our study. There is also a contradiction with the study by Decostere et al. [9], in which study one highly virulent

Table 2
Difference between colony morphology types 1, 2 and 4 formed by *Flavobacterium columnare* genetic groups E, G and H (see also Table 1) in virulence on rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings (Kaplan–Meier survival analysis, pairwise comparisons, Mantel Cox).

Genetic group and colony type	Difference in virulence: *P*-values				
	E1	E2	G2	G4	H2
E2	0.000[a]				
G2	0.000[a]	0.587			
G4	0.000[a]	0.787	0.778		
H2	0.000[a]	0.002[a]	0.007[a]	0.004[a]	
H4	0.000[a]	0.070	0.197	0.119	0.081

[a] Statistically significant difference in virulence.

F. columnare strain was more adherent on the gill tissue of black mollies (*Poecilia sphenops*) than a less virulent strain. Decostere et al. [9] did not provide any further information on the bacterial colonies, so comparison between their and our study is more difficult to make. In our study, the results from eight genetically different strains, representing high, intermediate and low virulence strains, suggest that adherence capacity is not the main factor defining virulence in *F. columnare*. Further, the adhesion factors of *F. columnare* in polystyrene adhesion are different from those in gill tissue adhesion.

In summary, we suggest, that strong adhesion capacity is not the defining virulence factor of *F. columnare*. Thus, some other virulence factors, such as chondroitin AC lyase activity [7] or features not yet found, are needed for pathogenesis in this bacterium. In addition, rhizoid colony morphology may also be related to virulence. This has already been shown in *F. psychrophilum*, when loss of gliding motility led to decreased virulence [43]. Moreover, in addition to *F. psychrophilum* [27], relation between rhizoid colony formation and gliding motility has been shown on *F. johnsoniae* [26,44]. However, our observation is based only on one *F. columnare* strain (E1), but the virulence experiment suggests similar gliding motility–virulence relationship as found in *F. psychrophilum*. To confirm this, more studies on the effects of colony morphology on bacterial virulence are needed.

4. Materials and methods

4.1. Bacterial strains and isolation of colony types

F. columnare type strain F (NCIMB 2248^T) and Finnish strains A–E and G–H isolated from diseased fish at different fish farms (see Ref. [10]) and exhibiting different degrees of virulence [7], were stored in −80 °C in 500 μl stocks containing 10% fetal calf serum (FCS, Gibco, BRL Co.) and 10% glycerol, thawed and inoculated to 10 ml of Shieh broth [5] (broth I). Cultures were grown at room temperature (RT) under constant shaking (120 rpm) to late log phase (23 h), after which the cultures were plated on Shieh agar (plate I). Plate cultures were grown for 3 days, different colony types isolated with a loop and inoculated to 10 ml of Shieh broth (broth II). Cultures were plated as previously (plate II), grown for 4 days, different colony types isolated and grown in Shieh broth (broth III) and plated (plate III) as previously. From broth III (broth II in case of strain F), stocks were made and stored in −80 °C. Plate I cultures were photographed using stereo microscope on days 2, 3 and 6, plate II cultures on days 2 and 4, and plate III cultures on days 2, 3, 4, 7, 9, 10, 17 and 22 after cultivation. For strain F only plate I and II cultures were made, and they were photographed on days 4, 7, 9, 10, 17 and 22 after cultivation. Several consecutive cultivations were made in order to check whether the colony types retained their morphology. For subsequent analyses, bacterial stocks made from broth III (broth II in case of strain F) were thawed and grown in 10 ml of Shieh broth to late log phase. Colony formation on another medium, AO broth and AO agar [45], was also tested. Experiment on AO medium was conducted as follows: *F. columnare* strains mentioned above were thawed, inoculated to 10 ml of AO broth and grown at RT under constant shaking (100 rpm) for 42 h, after which the cultures were plated on AO agar. Agar cultures were examined for colony formation using stereo microscope on days 2, 5, 7, 19 and 21 after cultivation. Also the colony types originally formed on Shieh agar (see Table 1) were cultivated in AO broth and on AO agar plates and plate cultures were examined as above.

4.2. Adhesion on polystyrene

Optical densities (OD) of bacterial cultivations at 595 nm were measured with micro plate reader (Multiskan Plus, Labsystems, Vantaa, Finland) and set to OD 0.260 in 200-μl volume, corresponding to 3.0×10^8 colony forming units (CFU) ml^{-1} of bacteria. For adhesion, 100 μl of each colony type culture and *Escherichia coli* strain JM109 as a control was inoculated onto 96-well polystyrene plates (Immuno Plate, Maxisorp, Nunc Co., Denmark) in six replicates. Another plate with four replicate wells of each colony type was used to detect bacterial growth rate during adhesion experiment. Bacteria were allowed to adhere on the plate for 2 h at RT. Relative adherence of bacterial cells was determined by the method described by Álvarez et al. [43]. Adhesion capacities were determined from OD values at 595 nm after crystal violet staining of adhered bacteria. ODs of growth controls at 595 nm were measured in the beginning and after the adhesion incubation.

4.3. Adhesion on gill tissue

The outermost gill arches (mean weight 30 mg) were taken from 12 rainbow trout fry (mean weight 23 g) and exposed to broth cultures of four *F. columnare* strains (C, E, G and H) representing colony types 1, 2 and 4 (C1, E1, E2, G2, G4, H2 and H4, see Table 1). ODs of bacterial cultivations were set to 0.119 (corresponding to 1.29×10^8 CFU ml^{-1}). One gill arch per treatment was placed on a petri dish and 15 ml of bacterial culture was added. Shieh broth was used as a negative control. For each bacterium, three replicate dishes were made. Bacteria were allowed to adhere on gill tissue for 2 h at RT under constant shaking (50 rpm). After adhesion, gill arches were rinsed with 10 ml of sterile distilled water and homogenized in eppendorf tubes containing 300 μl phosphate-buffered saline (PBS). From homogenate 1:10 serial dilutions were made in PBS, and 100 μl of pure homogenate and each dilution were plated on Shieh agar containing tobramycin, 1 μg ml^{-1} [4]. Plates were incubated at RT for 2 days after which plate counts were made and the adhesion capacities of the colony types determined from CFU ml^{-1} values.

4.4. ARISA (automated ribosomal intergenic spacer analysis)

Genomic DNA was extracted from colony types grown in Shieh broth using microbial DNA isolation kit (MOBIO). ARISA was conducted as previously [10] with following modifications: reaction mixtures contained 1.0 μM of each primer, 0.2 mM of dNTPs, 1× Biotools DNA polymerase buffer (Biotools), and 0.3 U of Biotools DNA polymerase (Biotools). The total reaction volume in each PCR tube was 30 μl plus 1.0 μl of template DNA, and the temperature of an initial denaturation step 95 °C.

4.5. AFLP (amplified fragment length polymorphism)

Genomic DNA of colony types was extracted as described above and subjected to AFLP based on Vos et al. [46]. Genomic DNA was digested successively with two restriction enzymes, EcoRI and MseI. Reaction solution (total volume 5.1 μl per sample), containing 2.0 U of EcoRI (Fermentas), 2.0 U of MseI (Fermentas), 0.5 μl of R+ buffer (Fermentas) and 4.2 μl DNA (ca. 20 ng), was incubated first at 37 °C for 1 h (for EcoRI digestion) and then at 65 °C for 2 h (for MseI digestion). By heating at 95 °C for 5 min and then cooling slowly, double stranded adapters for restriction sites were hybridized from single stranded EcoRI forward and EcoRI reverse (5.0 μM suspension), and MseI ad1 and MseI ad2 (50 μM suspension) adapters. These two stranded adapters were ligated at the ends of restriction fragments by incubating ligation solution (total volume 10 μl per sample), including 1.0 U of T4 DNA ligase (Fermentas), 1× T4 ligation buffer (Fermentas), 0.1 μM of EcoRI adapter, 1.0 μM of MseI adapter, 3.4 μl of sterile distilled water and 5.0 μl of digestion product, at RT for 1 h. The ligation products were used as templates in PCR, in which adapter specific primers were used: one reaction

was done using primer pair MseI-0/EcorRI-A-IRD700 and another using MseI-0/EcorRI-ACT-IRD800, in which A and ACT stand for additional selective nucleotides and 0 for no selective nucleotide. Twenty microliters of PCR reaction mixture, containing 0.25 μM of each primer, 1.0 mM of $MgCl_2$ (Fermentas), 0.08 mM of dNTPs, 0.2 U of Taq DNA polymerase (Fermentas), 1 × Tag DNA polymerase buffer [$(NH_4)_2SO_4$ added] (Fermentas), 11.2 μl of sterile distilled water, and 3.0 μl of template, was made per each sample. PCR was run according to following program: initial denaturation step at 94 °C for 2 min, 13 cycles of amplification I (94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min), 24 cycles of amplification II (94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min), and final elongation step at 72 °C for 10 min. The AFLP was run with a LI-COR 4200 automatic sequencer (LI-COR BioTech) in 6% Long Ranger denaturing polyacrylamide gel (FMC Bioproducts), and fragment length data was analyzed using IrfanView 3.97 free software.

4.6. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) of whole cell proteins

Whole cell proteins were extracted from broth cultures of colony types according to protocol by Tan et al. [47] with slight modifications. Bacterial culture, 100 ml, was centrifuged at 4332 × g for 10 min. The pellet was washed once with 50 ml of 10 mM Tris-HCl buffer, pH 7.6, and resuspended in 20 ml of the buffer. The extract was sonicated on ice (Branson Digital Sonifier®, Branson Ultrasonic Corporation, Danburry, Connecticut, USA) for 1 min (1 s pulse with 2 s interval), and 500 μl of sonicate was suspended in an equal volume of 2× electrophoresis treatment buffer and stored in −20 °C until analyzed. Prior electrophoresis, the samples were heated at 100 °C for 2 min and placed on ice. Discontinuous polyacrylamide gel, 16 cm long and 1.5 mm thick, was used. Samples (50 μl) were pipetted into wells, and electrophoresis was run first with 120 V for 1 h, then with 180 V for 4 h, and finally with 200 V for 1 h. The protein patterns were visualized by staining with PageBlue™ Protein Staining Solution (Fermentas).

4.7. Challenge experiment

Three *F. columnare* strains (E, G and H) representing colony types 1, 2 and 4 (E1, E2, G2, G4, H2 and H4, see Table 1), were tested for their virulence in rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings. Strain E has previously been defined as high virulence, G as intermediate virulence, and H as low virulence strain [7]. These strains were chosen because in the present study it was not possible to carry out an experimental set up with all colony types formed by all genetic groups. Fish, (mean weight 6.0 g) originated from a stock of a fish farm in Central Finland, and were acclimated in laboratory conditions for 3 weeks before the experiment. Bacterial infection of fish was carried out under constant aeration in 10-l plastic aquaria, 25 fish in each aquarium, with well water containing 6.5×10^6 CFU ml^{-1} of bacteria. Each bacterial infection was done in triplicate, a total of 75 fish per treatment. Fish treated with Shieh broth without bacterial inoculate served as negative control. The water temperature was 25 °C and infection time 2 h. After infection, the fish were dipped in pure well water and transferred at random into clean, aerated, 10-l plastic flow trough-aquaria ($T = 25$ °C). Fish were monitored for 8 days by 12-h intervals for signs of disease and mortality. Bacterial cultivations from skin and gill lesions of up to ten dead fish per treatment were made on Shieh agar. At the end of the experiment cultivations were made from the trunk kidney of four fish per treatment. Cumulative mortality of fish was used as an indicator of bacterial virulence.

4.8. Statistics

Differences between adhesion capacities of *F. columnare* strains forming colony types 1–4 were analyzed using SPSS 14.0 software. In the polystyrene adhesion experiment, comparisons between OD values of each strain and their colony types were made using non-parametric Kruskal–Wallis test. Means of OD values of each colony type were calculated and compared using one-way ANOVA. CFU counts of gill tissue adhesion were analyzed using one-way ANOVA. Data from the bacterial challenge experiment was analyzed using Kaplan–Meier survival analysis. *P*-values below 0.05 were considered significant.

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