Bacteriophage Resistance Affects *Flavobacterium columnare* Virulence Partly via Mutations in Genes Related to Gliding Motility and Type IX Secretion System

Heidi M. T. Kunttu[1]#, Anniina Runtuvuori-Salmela[1], Krister Sundell[2], Tom Wiklund[2], Mathias Middelboe[3], Lotta Landor[2*], Roghaieh Ashrafi[1], Ville Hoikkala[1], Lotta-Riina Sundberg[1]

[1]Department of Biological and Environmental Science and Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland

[2]Laboratory of Aquatic Pathobiology, Åbo Akademi University, Turku, Finland

[3]Department of Biology, Marine Biological Section, University of Copenhagen, Helsingør, Denmark

Running Head: Phage Resistance in *Flavobacterium columnare*

Keywords: bacteriophage, colony morphology, *Flavobacterium columnare*, gliding motility, mutation, phage resistance, type IX secretion system, virulence

#Address correspondence to Heidi M. T. Kunttu, heidi.kunttu@jyu.fi

*Present address: Lotta Landor, Department of Biological Sciences, University of Bergen, Bergen, Norway

Abstract

Increasing problems with antibiotic resistance has directed interest towards phages as tools to treat bacterial infections in the aquaculture industry. However, phage resistance evolves rapidly in bacteria posing a challenge for successful phage therapy. To investigate phage resistance in the fish pathogenic bacterium *Flavobacterium columnare*, two phage-sensitive, virulent wild-type isolates, FCO-F2 and FCO-F9, were exposed to phages and subsequently analyzed for bacterial viability and colony morphology. Twenty-four phage-exposed isolates were further characterized for phage resistance, antibiotic susceptibility, motility, adhesion and biofilm formation on polystyrene surface, protease activity, whole genome sequencing and virulence against rainbow trout fry. Bacterial viability first decreased in the exposure cultures, subsequently increasing after 1-2 days. Simultaneously, the colony morphology of the phage-exposed isolates changed from original rhizoid to rough. The rough isolates arising in phage exposure were phage-resistant with low virulence, whereas rhizoid isolates maintained phage sensitivity, though reduced, and high virulence. Gliding motility and protease activity were also related to the phage sensitivity. Observed genetic mutations in phage-resistant isolates were mostly located in genes coding for type IX secretion system, a component of the flavobacterial gliding motility machinery. However, there were mutational differences between individual isolates, and not all phage-resistant isolates had genetic mutations. This indicates that development of phage resistance in *F. columnare* probably is a multifactorial process including both genetic mutations and changes in gene expression. Phage resistance may not, however, be a challenge for development of phage therapy against *F. columnare* infections, since phage resistance is associated with decrease in bacterial virulence.

Importance

Phage resistance of infectious bacteria is a common phenomenon posing challenges for development of phage therapy. Along with growing World population and need for increased food production, constantly intensifying animal farming has to face increasing problems of infectious diseases. Columnaris disease, caused by *F. columnare*, is a worldwide threat for salmonid fry and juvenile farming. Without antibiotic treatments, infections can lead to 100% mortality in a fish stock. Phage therapy of columnaris disease would reduce a development of antibiotic-resistant bacteria and antibiotic loads by the aquaculture industry, but phage-resistant bacterial isolates may become a risk. However, phenotypic and genetic characterization of phage-resistant *F. columnare* isolates in this study revealed that they are less virulent than phage-sensitive isolates and thus not a challenge for phage therapy against columnaris disease. This is a valuable information for the fish farming industry globally when considering phage-based prevention and curing methods for *F. columnare* infections.

Introduction

Aquaculture has a central role in supporting the increasing demand for high quality protein and healthy food. However, the use of chemotherapy in disease treatment in the industry has led to increased resistance of disease-causing agents to commonly used antibiotics (1, 2). Further, in the face of climate warming, production of protein with smaller carbon footprint is of increasing importance. This has put a pressure on aquaculture industry to increase efficiency in food production, which also means developing more effective ways to fight infectious diseases in intensive farming including reduction the use of antibiotics. Although vaccines against many microbial diseases are in use globally in aquaculture, there are still

many diseases with no potent immunization method available (3). This applies especially to infections of fish fry, where efficiency of vaccination is poor due to lack of development of fish secondary immunity at the early life stage.

One of these diseases affecting fry is caused by the fish pathogenic bacterium *Flavobacterium columnare*, the infectious agent of columnaris disease. Columnaris infections cause extensive losses in farmed salmonid fry and juveniles, populations of different catfish species and ayu (*Plecoglossus altivelis*) around the world in water temperatures above 18 °C. The only effective curing method is antibiotic treatment. However, infections often occur repeatedly and may cause up to 100% mortality in rainbow trout fry populations if not treated, thus causing major economic losses to the industry (4, 5). In addition, elevated water temperatures due to warmer summers in the recent years are suggested to enhance virulence development in *F. columnare* (5). Although antibiotic resistance in this bacterium is not yet as severe problem as in related pathogens, e.g. *Flavobacterium psychrophilum* (6, 7) or *Vibrio* species (8, 9), strains that have acquired resistance towards commonly used antibiotics already exist (10).

Bacteriophages (phages) are viruses that specifically infect their host bacteria, without harming the surrounding microbial community (reviewed in 11). Among the alternatives to traditional antibiotics, phage therapy, i.e. the use of phages against bacterial infections, has demonstrated a strong potential for controlling disease outbreaks in aquaculture (e.g. 12-14). Promising results have been gained also in phage therapy trials of Flavobacterial infections. In a study by Castillo et al. (15), phage treatment reduced the mortality of *F. psychrophilum*-infected Atlantic salmon (*Salmo salar*) by 60 % and rainbow trout (*Oncorhynchus mykiss*) by 67 %. In studies with columnaris infections, mortality of zebra fish (*Danio rerio*) and

98 rainbow trout were reduced by 100 % and nearly 42 %, respectively, in the presence of
99 phages (16). In addition, pre-colonization of fish with phage significantly slowed down the
100 infection and reduced the mortality of rainbow trout (17).
101
102 One of the biggest challenges for phage therapy is the imposed selection for phage resistance
103 among phage-exposed bacteria. Bacteria have developed a variety of phage defence
104 strategies, including surface modification and cell aggregation, inactivation of intruding
105 phage DNA by Restriction-Modification and CRISPR-Cas systems, proteolytic digestion of
106 phage particles, and quorum sensing regulation of phage receptor expression (e.g. 18- 20).
107 The prevalence and control of these resistance mechanisms depend specifically on the phage-
108 bacterium interaction, on the type and function of the receptor, and the costs of engaging the
109 different mechanisms under various environmental conditions. In many pathogenic bacteria
110 the cell surface molecules are functioning as virulence factors, and phage-driven changes in
111 these structures leading to phage resistance often lead to simultaneous reduction in virulence
112 (21). This trade-off has been detected also among several bacterial fish pathogens, e.g. in
113 *Pseudomonas plecoglossicida* (22), *F. psychrophilum* (23) and *Vibrio anguillarum* (24).
114
115 Exposing *F. columnare* to phages has been observed to cause a change in colony
116 morpohotype from the ancestral rhizoid form to rough, which is associated with loss of
117 gliding motility and virulence (25-27). Since a change in colony morphology and loss of
118 virulence have been observed previously also by deletion of genes in the Type IX secretion
119 system involved in gliding motility of *F. columnare* (28), it is likely that mutations in this
120 secretion system are also linked with phage resistance in *F. columnare* (29). Yet, the exact
121 mechanisms by which phages cause the colony morphology change in *F. columnare*, and the
122 functional implications for the bacteria have not been previously explored.

Understanding the mechanisms and consequences of phage resistance in the target bacteria is central for development of successful phage therapy. Thus, in this study, we exposed two *F. columnare* isolates (FCO-F2 and FCO-F9) separately to three different phages, and studied infection dynamics, bacterial viability and colony morphology, and isolated phage-resistant bacteria. Twenty-four phage-exposed and no-phage control isolates were further characterized for their phage resistance, antibiotic susceptibility, motility, adhesion and biofilm formation on polystyrene surface, protease (elastinase, gelatinase and caseinase) activity, virulence on rainbow trout fry, and whole genome sequence. Our results show, that if phage resistance in *F. columnare* is gained via surface modification leading to morphotype change, virulence decreases. However, if the colony morphology remains rhizoid, the isolates remain highly virulent with reduced sensitivity to phage compared to the ancestral wild-type strain.

Results

Isolates from phage-exposures: growth, colony morphology and phage resistance

In all phage-exposure cultures of FCO-F2, there was a strong initial phage control of the host population during the first day in all the phage-exposed cultures compared with control culture without phages (Figure 1a). After this, the bacterial density started to recover. The phage-free cultures grew exponentially during the first day, after which they reached a plateau phase. Along with the population decline on day 1, bacterial colony morphotype changed from ancestral rhizoid to rough (Figure 2). From day 1 onwards, more than 88% of the colonies formed by phage-exposed bacterial isolates were rough, the amount reaching at

148 least 97% at the end of the experiment (Figure 1c). In addition, in FCOV-F25 exposure, few
149 soft colonies were observed on day 2 (Figure 2), and in no-phage control cultures, some
150 rough colonies appeared among the prevailing rhizoid ones.
151
152 FCO-F9 showed slightly different growth dynamics. The bacterial population size increased
153 exponentially during the first day in all cultures (Figure 1b), but decreased drastically on day
154 2 in response to phage exposure, and then reached exponential growth again. The phage-free
155 cultures reached a plateau phase on day 2, after which the amount of culturable bacteria
156 decreased. From the day 2 population crash and onwards, more than 85% of the colonies
157 formed by phage-exposed bacteria had rough morphology (Figure 1d). At the end of the
158 experiment, more than 98% of the colonies where rough. In FCOV-F13 exposure, a few
159 rough colonies were observed already on day1 and some soft colonies on days 2 and 3. In no-
160 phage control cultures, some (4 %) rough colonies appeared among the rhizoid ones on day 3.
161
162 Out of 189 colonies collected from phage exposures, 20 phage-exposed and 4 no-phage
163 control isolates were characterized further (Table 1). Of these isolates, the no-phage control
164 isolates all formed rhizoid colonies similar to their wild-type parent, phage-sensitive isolates
165 FCO-F2 and FCO-F9. Most of the phage-exposed isolates were of rough colony morphology,
166 but F2R58, F2R66 and F9R56 had rhizoid, and F9R69 soft colony morphology.
167
168 All the phage-exposed rough isolates were resistant to all the phages used to infect the
169 ancestor wild-type bacteria (Table 1). In addition, in some cases, phage caused inhibition of
170 bacterial growth, considered as phage resistance because no clear plaques due to phage
171 infection were detected. The rhizoid phage-exposed isolates turned out to be partly phage-
172 resistant with a 5.5 X 10^5 to 11 X 10^5-fold reduction in phage susceptibility compared to the

wild type isolates, depending on the specific phage (results not shown). Throughout this paper, these isolates with decreased phage sensitivity are grouped together with the phage-sensitive isolates.

Antibiotic susceptibility

All isolates showed antibiotic susceptibility patterns similar to the parent wild-type isolates, and no notable differences were observed (Figure S1 and Table S1).

Motility, adhesion and biofilm formation

Phage-sensitive bacteria forming rhizoid colonies were significantly more motile (determined as colony spreading) than phage-resistant rough or soft morphotypes, irrespective of isolation history (F2-isolates: $P < 0.001$, Oneway ANOVA, LG10 transformation; F9-isolates: $P \leq 0.004$, Mann-Whitney test) (Figure 3).

Compared to the parent wild-type FCO-F2 isolate, there was a large variability on the adhesion capacity of individual phage-resistant F2-isolates (Figure 4a). Phage susceptibility (rhizoid vs. rough colony type) or phage used in the co-culture experiment did not influence bacterial adhesion capacity ($P = 0.3$: Mann-Whitney test and $P = 0.564$: Kruskal-Wallis test, respectively).

Most of the individual phage-exposed and no-phage control F2-isolates had significantly lower biofilm forming capacity than in parent wild-type FCO-F2 ($P \leq 0.017$: Oneway ANOVA, LDS multiple comparisons, square root transformation) (Figure 4c). Still, there was

198 no statistical difference in biofilm formation between phage-sensitive rhizoid and resistant
199 rough morphology F2-isolates ($P = 0.062$: Oneway ANOVA).
200
201 Again, the bacterial strain F9 behaved differently compared to F2. In contrast to the phage-
202 resistant F2-isolates, the phage-resistant rough and soft morphology F9-isolates had
203 significantly lower adherence than sensitive rhizoid isolates ($P < 0.001$: Oneway ANOVA,
204 LDS multiple comparisons, square root transformation) (Figure 4b). In addition, isolates
205 exposed to phages isolated in 2017, FCOV-F13 and FCOV-F45, had significantly lower
206 adhesion capacity than in isolates exposed to FCL-2 isolated in 2009 ($P < 0.001$: Mann-
207 Whitney test). This may indicate phage FCL-2 uses different phage receptor (see later).
208
209 In contrast to adhesion ability, biofilm forming capacity of the most of the individual phage-
210 exposed and no-phage control F9-isolates was significantly higher compared to wild-type
211 parent isolate ($P \leq 0.004$: Oneway ANOVA, LDS multiple comparisons) (Figure 4d). F9R69
212 with soft colony morphology did not form any biofilm and thus excluded from the multiple
213 comparisons. Phage-resistant rough F9-isolates had significantly higher biofilm forming
214 capacity than sensitive rhizoid morphotypes ($P < 0.001$: Oneway ANOVA, square root
215 transformation).
216
217 *Protease activity: elastinase, gelatinase and caseinase*
218
219 Elastinase activity was detected in the wild-type, and all the phage-sensitive rhizoid FCO-F2
220 isolates and one resistant rough F2-isolate (clear zone ratio > 1), whereas all remaining
221 resistant, rough morphology isolates, had completely lost the ability to degrade elastin
222 (Figure 5a). There were no differences in elastinase activity between the elastinase positive

223 isolates ($P = 0.843$: Oneway ANOVA). Elastinase activity was not detected in any of the F9-
224 isolates (clear zone ratio = 1) (Figure 5b).
225
226 There were variations in gelatinase activity between individual F2- and F9-isolates (Oneway
227 ANOVA, LDS multiple comparisons) (Figure 5c and d). However, among both F2- and F9-
228 isolates, gelatinase activity of phage-resistant rough morphotypes was lower than that of
229 sensitive rhizoid morphotypes (F2-isolates: $P = 0.018$, Oneway ANOVA, exponential
230 transformation; F9-isolates: $P < 0.001$, Oneway ANOVA). Two of the phage-exposed F9-
231 isolates (F9R69 and F9R78) did not have any gelatinase activity and were thus excluded from
232 the multiple comparisons
233
234 Less variation in caseinase activity between individual isolates was observed (Oneway
235 ANOVA, LDS multiple comparisons) (Figure 5e and f), and phage-sensitive rhizoid and
236 resistant rough F2-isolates did not differ from each other ($P = 0.058$: Oneway ANOVA. On
237 the other hand, caseinase activity of phage-resistant rough and soft F9-isolates was lower
238 than that of sensitive rhizoid isolates ($P = 0.007$: Oneway ANOVA).
239
240 *Virulence*
241
242 Rainbow trout fry were exposed to wild-type, phage-exposed and no-phage control isolates,
243 and all of them caused mortality during 24 h (Figure 6). The phage-sensitive rhizoid
244 morphotypes were most virulent, causing 100 % mortality, whereas resistant rough and soft
245 morphotypes were less virulent, causing 46.7 % mortality at highest (except for phage-
246 resistant rough morphotype F2R70, which caused 100 % mortality). Mortality of control fish
247 was 15 %, but no bacterial growth was observed from these fish. However, *F. columnare*

248 growth was observed from all the fish exposed to bacteria. Colony morphotype of the
249 bacterial isolates did not change during the infection.
250
251 When comparing the data according to the phage susceptibility and thus colony morphology,
252 cumulative mortality of fish infected with phage-sensitive rhizoid morphotypes, irrespective
253 of if they were wild-type, phage-exposed or no-phage control isolates, was significantly
254 higher than mortality caused by phage-resistant rough or soft morphotypes among both F2
255 and F9 isolates ($P < 0.001$, Kaplan-Meier Survival Analysis). Also, the estimated survival
256 time (Kaplan-Meier Survival Analysis) was shortest in fish infected with sensitive rhizoid
257 isolates (Figure 6). In case of F2-isolates, mortality caused by phage-resistant rough isolates
258 was also significantly higher than mortality of control fish, but mortality caused by resistant
259 rough and soft F9-isolates did not differ from each other or from the control fish mortality.
260 Mortality caused by rhizoid phage-sensitive F2 isolates started to peak at 12 hours post
261 infection (p.i.) and in F9 at 16 hours p.i. ($P < 0.001$, Kaplan-Meier Survival Analysis), but
262 between rough phage-resistant F2 and F9 isolates the mortality patterns were more similar
263 starting to increase slowly at 2-3 hours p.i. ($P = 0.217$, Kaplan-Meier Survival Analysis).
264 However, there were differences in cumulative mortalities caused by individual isolates in
265 each morphology group (Data set S1).
266
267 *Whole genome sequencing*
268
269 Genome data of wild-type *F. columnare* isolates FCO-F2 and FCO-F9 is presented in Table
270 2.
271

Genomic comparisons between F2 wild type and phage-exposed isolates revealed a limited number of genomic changes. In seven out of 11 isolates, single mutation leading to formation of wrong or truncated proteins was observed in the phage-resistant mutants (Table 3). Notably, the majority of the mutations were located in genes coding for gliding motility proteins *gldB* (F2R67), *gldN* (F2R72) and *sprA* (F2R60, F2R64, F2R65, F2R74). Isolate F2R70 had one nucleotide insertion in OmpH family outer membrane protein coding gene. Three isolates (F2R62, F2R66, F2R68) did not show any genomic changes relative to the wild type. In isolate F2R58 with decreased phage sensitivity, one nucleotide change in *rlmF* gene (coding for rRNA large subunit methyltransferase F) did not lead to amino acid change. No mutations were observed in the no-phage control isolates. At certain points of ribosomal RNA operons in all phage-exposed and no-phage control isolates, and also in a 736 221 bp sequence (hypothetical protein coding sequence in wild-type FCO-F2 genome used as a reference) in phage-exposed isolates F2R66 and F2R68, there was a poor coverage of reads leading to unclear sequences, which prevented detection of possible mutations in this region.

In F9 phage-exposed isolates, one or two mutations per isolate in all the other isolates, except for F9R58, were observed (Table 4). Mutations in isolates exposed to FCOV-F45 had insertions whereas FCOV-F13 exposed isolates had deletions or single nucleotide chances in genes coding for gliding motility proteins *gldG* (F9R72), *gldM* (F9R64, F9R69, F9R78) and *gldN* (F9R69, F9R75), leading to formation of wrong or truncated proteins. Interestingly, in the isolate F9R69 (exposed to FCOV-F13) with a soft colony type, a deletion of genomic region of 4 701 bp was observed, spanning over gliding motility genes *gldM* and *gldN,* and sequences coding for FAD-binding oxidoreductase, DUF3492 domain-containing protein and a hypothetical protein (Figure 7).

297 On the contrary, no mutations in gliding motility genes were observed in F9 isolates exposed
298 to FCL-2, but instead, two of these isolates had one nucleotide change in
299 DegT/DnrJ/EryC1/StrS family aminotransferase and DUF255-domain containing protein
300 (F9R56), and cystathionine gamma-synthase (F9R61) coding genes, leading to either one
301 amino acid change or truncated protein. No mutations were observed in no-phage control
302 isolates. Around 2 000 620 bp (hypothetical protein coding sequence in B185 genome used as
303 a reference), there was a poor coverage of reads leading to unclear sequence in both wild type
304 FCO-F9, phage-exposed and no-phage control isolates, which prevented detection of possible
305 mutations in this region.
306
307 **Discussion**
308
309 Phage therapy is seen as an attractive option to treat and prevent bacterial diseases, but the
310 development of phage resistance in target bacteria is considered as one of the main problems
311 related to the use of phages. Our results describe the selection for phage resistance in two
312 different *F. columnare* isolates upon exposure to six specific phages. We show that phage
313 resistance is associated with reduction in virulence and virulence-related phenotypic changes
314 in the bacterium. Our genetic data indicate that in most cases phage resistance is caused by
315 surface modifications, often related to the type IX secretion system connected to
316 flavobacterial gliding motility machinery. Mutations in the genes coding for an outer
317 membrane protein or genes related to gliding motility seem to be phage specific and likely
318 prevent phage attachment, possibly in a phage specific manner, and lead to morphology
319 change and loss of virulence.
320

In the present study, phage-exposure caused significant changes in bacterial phenotypic characteristics (motility, adhesion, protein secretion and virulence - details below) leading to phage resistance. In most isolates, these changes could be linked to changes in gliding motility-related genes. Flavobacteria show gliding motility on surfaces (29), and mutations in any of the genes coding for gliding motility machinery proteins have been shown to lead to loss of motility (e.g. 30, 31). Gliding is also connected to virulence, since part of the gliding motility machinery (GldK, GldL, GldM, GldN, PorV, SprA, Spr E, SprF and SprT) is used as a type IX secretion system found in Bacteroidetes (28, 32). Indeed, phage resistance due to loss of motility has been linked with decreased virulence in *F. columnare* also previously (27), and *F. columnare gldN* mutants have been shown to exhibit both decreased proteolytic and chondroitinase activity, and virulence on rainbow trout (28). Similarly, phage resistance was associated with loss of motility and mutations in genes related to cell surface properties and gliding motility in *F. psychrophilum* (23) and in *F. johnsoniae* (31, 33). Together, the results suggest that the type IX secretion system is a key target for infection by a wide range of phages and across the Flavobacterium genus, and that mutations leading to morphology changes and loss of motility is a general response to phage exposure in this bacterial group.

Exposure to a specific phage led to different mutations in gliding motility genes in different *F. columnare* isolates, as also seen in phage-resistant *F. psychrophilum* (23), indicating that several genes are involved in phage attachment and infection of *F. columnare* phages. Furthermore, genomic analysis of one soft colony isolate revealed a large deletion (4 701 bp), spanning over two gliding motility genes. However, although all rough colony forming isolates were phage-resistant, not all these isolates (F2R62, F2R66, F2R68 and F9R58) had mutations in genes coding for proteins related to gliding motility, or elsewhere in their genome. This may indicate that development of phage resistance and colony morphology

346 change are also influenced by gene expression or epigenetic modifications, leading to
347 variation in colony morphology, as suggested previously (34). For example, in *Bordetella*
348 spp, phage resistance is regulated via phase variation in virulence related factors, such as
349 some adhesins, toxins and type III secretion system (reviewed in 35). Interestingly, isolates
350 exposed to FCL-2 did not have mutations in gliding motility related genes, suggesting that
351 FCL-2 uses other receptors for infection of *F. columnare* than the other phages. FCL-2
352 differs genetically from other phages infecting genetic group G bacteria (This article was
353 submitted to an online preprint archive [36]), supporting this suggestion.
354
355 Generally, point mutations and changes in receptor expression enable a rapid and efficient
356 response of bacterial populations to phage exposure. However, the large phenotypic costs of
357 mutational derived phage resistance observed in *F. columnare* in this study suggest that these
358 mutations may be dynamic and most probably also rapidly reverting back to the sensitive
359 form in *F. columnare*. Indeed, reversion of both phage-driven and spontaneously formed
360 rough colony types back to rhizoid has been observed to happen in *F. columnare* subcultures
361 (27). Various mechanisms to regain phage resistance have been found also in fish pathogenic
362 *F. psychrophilum* (23) and *V. anguillarum* (24), in which a rapid reversion back to phage-
363 sensitive phenotype has been shown to occur. This sort of dynamics in phage resistance has
364 also been observed in a human symbiont *Bacteroides thetaiotaomicron* (37), suggesting that
365 the phenomenon may be common among wide variety of bacteria.
366
367 Phage-exposed *F. columnare* isolates F2R56, F2R66 and F9R58 did not respond to phage
368 infection with surface modifications, but maintained their original rhizoid colony morphotype
369 and high virulence. These rhizoid isolates were not completely resistant to phage, although
370 phage infection efficiency dropped markedly (up to a million-fold decrease), suggesting some

371 other mechanism for reducing infection efficiency. *F. columnare* has two functional CRISPR
372 systems, which have been shown to adapt under phage exposure at fish farms (38). However,
373 we did not observe additional CRISPR spacers in whole genome sequencing. The same was
374 observed in phage exposed *F. psychrophilum* isolates in which no differences to the wild-
375 type strain's CRISPR composition were found (23). In our experience, CRISPR adaptation in
376 *F. columnare* requires different experimental set-up with longer co-culture time in low
377 nutrient medium, followed by enrichment in high-nutrient medium (This article was
378 submitted to an online preprint archive [39]). Thus, the decreased phage sensitivity of rhizoid
379 phage exposed isolates most probably is a consequence of yet unknown functions which need
380 to be studied in the future.
381
382 In addition to type IX secretion system, also type I and VI secretion systems are known to
383 function in *F. columnare* (40). Possible secretion of virulence related factors through type I
384 and VI secretion systems in *F. columnare* could be one of the reasons why also rough phage-
385 resistant isolates caused some mortality in fish, and explain their gelatinase and caseinase
386 activity despite morphology change. It has also been shown recently, that virulence of *F.*
387 *columnare* increases in the mucus and with increasing mucin concentration (17). As the
388 mucus-covered fish surface is the main infection route of *F. columnare*, it is probable that
389 some *F. columnare* virulence factors, such as proteinase activity, are expressed differently in
390 growth media compared to the *in vivo* infection situation. This possible differential
391 expression could also explain the mortality caused by phage-resistant rough isolates.
392
393 The ability to adhere and form biofilm has a major role in bacterial infections and in
394 colonizing niches (41). In *F. columnare*, adhesion and biofilm forming capacity may have a
395 central role in their persistence in the farming environment (e.g. tanks and water systems)

(42), but also in establishing the first steps of infection on the fish surfaces (43). Our results indicate that *F. columnare* strains differ in their adherence and biofilm forming characteristics. Whereas phage exposure had no clear effect on the adhesion capacity of the F2-isolates, phage resistance led to decrease in biofilm forming capacity in most of the individual phage-resistant F2-isolates. This is in agreement with the systematic reduction in biofilm forming properties of phage-resistant *F. psychrophilum* relative to the wild type (23). Adhesion capacity of F9 phage-resistant isolates, on the other hand, was significantly lower compared to the wild-type parent isolate, but rough phage-resistant F9-isolates had significantly higher biofilm forming capacity compared to rhizoid sensitive isolates. These results partly differ from what we have found earlier (25, 26), most likely because in the previous studies the rough colonies were formed spontaneously, without phage exposure. Indeed, morphology of spontaneously formed rough colonies and these morphotypes' ability to move when cultured in low-nutrient media differ from rough morphotypes formed under phage exposure (27). However, together our results indicate, that since *F. columnare* phages are genetically group-specific, they might be using different receptors, which, in turn, causes differences in bacterial resistance mechanisms between genetic groups.

F. columnare infections are routinely treated by antibiotics at fish farms. In this study, phage resistance did not affect the antibiotic susceptibility of any of the isolates studied. Lack of association between development of antibiotic resistance and bacteriophage resistance has also been shown e.g. in *Escherichia coli* (44). Based on our results, phage resistance does not increase a risk of antibiotic resistance development, and thus, phage-therapy given as a cure or prophylactic treatment at fish farms most probably does not rule out the possible concomitant use of antibiotics as therapeutic agents against columnaris infections. Indeed, it was shown by using *P. fluorescence* as a model bacterium, that applying phages together

421 with antibiotic treatments may inhibit the evolution of antibiotic resistance in pathogenic
422 bacteria (45).
423
424 To summarize, our results show, that even though *F. columnare* rapidly develops phage
425 resistance under phage exposure, the arise of phage resistance does not pose a high risk for a
426 development of phage therapy against columnaris infections in rainbow trout. This is because
427 phage resistance leads to decrease in bacterial virulence, adherence to surfaces and protease
428 secretion. Based on our results with experiments with two genetically different wild-type
429 bacterial isolates, development and regulation of phage resistance in *F. columnare* is a
430 multifactorial process, partly affected by formation of mutations mainly in gliding motility
431 and type IX secretion system related genes, and partly by other defence mechanisms against
432 phages, the function of which needs to be studied in the future.
433
434 **Materials and methods**
435
436 *Bacterial and phage isolates*
437
438 Bacteria and phages used in this study were isolated from water samples collected from fish
439 farms during columnaris outbreaks (This article was submitted to an online preprint archive
440 [36]) (Table 5). Bacteria were confirmed as *F. columnare* by restriction fragment length
441 polymorphism (RFLP) analysis of 16S rRNA gene and classified into genetic groups by
442 RFLP of 16S-23S internal transcribed spacer (ITS) region (This article was submitted to an
443 online preprint archive [36]). All the six phages belong to the *Myoviridae* family and have
444 been characterised with respect to host range and genomic composition (This article was
445 submitted to an online preprint archive [36]).

446

447 *Bacterial cultures and phage lysates*

448

449 For phage exposure and virulence test, *F. columnare* isolates were inoculated from
450 cryopreserved (–80°C) stocks in modified Shieh-medium (46) and grown for 48 h at 25°C
451 with 120 rpm agitation. After this, subcultures were made in modified Shieh-medium and
452 grown for 24 h at 25°C with 120 rpm agitation. The optical density (OD) of the bacterial
453 broth suspensions was measured spectrophotometrically at 595 nm and adjusted to 5 X 10^5
454 colony forming units (CFU) mL^{-1} for phage exposures and 5 X 10^6 CFU mL^{-1} for virulence
455 experiment (based on previously determined OD/CFU relationship). For other test, *F.*
456 *columnare* isolates were cultured in TYES broth (47), washed in TYES broth by
457 centrifugation at 5310 X *g* for 15 min at 4°C, and cultures spectrophotometrically adjusted to
458 OD 0.6 at 520 nm (approximately 10^8 CFU mL^{-1}).

459

460 Phage lysates were produced using "double layer agar" -method (48) as follows: Three mL of
461 melted (47°C) top agar (0.5%) including 300 µL of 24-hour subculture of the host bacterium
462 and 100 µL of phage (tenfold dilutions in Shieh medium) was poured on Shieh agar and
463 grown for 48 h at 25 °C. Five mL of Shieh-medium was added on top of Shieh agar plates
464 with confluent lysis and incubated at 7°C for 12-18 h in constant agitation (90 rpm). The
465 lysates were collected, filtered (PES membrane, pore size 0.45 µm, Nalgene®), and stored at
466 +7°C or at –80°C with 20 % glycerol. For phage exposure, phage lysates were diluted with
467 Shieh medium to 5 X 10^5 plaque forming units (PFU) mL^{-1}.

468

469 *Phage exposure experiments and isolation of colonies*

470

Two phage-sensitive wild-type *F. columnare* isolates, the high-virulence FCO-F2 isolate (genetic group C) and the medium-virulence FCO-F9 isolate (genetic group G) (This article was submitted to an online preprint archive [36]), were each exposed to three phages in separate experiments with individual phages. Isolate FCO-F2 was exposed to phages FCOV-F2, FCOV-F5 and FCOV-F25, and isolate FCO-F9 to phages FCL-2, FCOV-F13 and FCOV-F45, in accordance with the host range of the phages. Cultures with only bacteria served as no-phage controls. The exposures were carried out in 20 mL of autoclaved fresh water (Lake Jyväsjärvi) in triplicate cultures under constant agitation (120 rpm) at 25ºC for three days at a multiplicity of infection (MOI) at inoculation of 1 (1 X 10^4 CFU and PFU mL^{-1}). The cultures were sampled every 24h for three days, by making a serial tenfold dilution of samples, and spreading on Shieh-agar plates. After up to 4 days of incubation at room temperature, CFUs and colony morphologies were determined from the plate cultures. Two to three colonies from each triplicate culture at each sampling point were picked, and pure-cultured directly on Shieh agar plates three times to get rid of any phage contamination. Colonies were then checked for phage resistance by spot assay on agar plates: bacterial laws on top agar were prepared as above and 10 µL of ten-fold diluted original phage lysates (used in initial exposures) were spotted on agar. After 48-h incubation at 25 °C, bacterial plates with no observed plaques or confluent lysis were considered as phage-resistant. Altogether 189 colonies from phage-exposed and no-phage control exposures were isolated from plate cultures. From this collection, 20 phage-exposed and 4 no-phage control isolates were selected for further analysis (Table 1).

The phage-exposed and no-phage control isolates were named according to the latter part of the wild-type bacterial host, a letter R for phage-exposed and S for no-phage control isolate, plus a running number for the isolated colony. For example, F2R2 is the second selected

496 phage-exposed colony of the *F. columnare* wild-type isolate FCO-F2. Correspondingly, the
497 second *F. columnare* isolate from no-phage control cultures was marked as F2S2. For
498 simplicity, wild-type FCO-F2 and all its subsequent isolates from the phage and control
499 exposures are commonly called F2-isolates in this paper. Correspondingly, wild-type FCO-
500 F9 and its subsequent isolates are called F9-isolates.
501
502 *Antibiotic sensitivity*
503
504 Changes in susceptibility of phage-exposed *F. columnare* isolates towards antibiotics was
505 tested using the Kirby-Bauer disc diffusion method (49) on diluted Mueller-Hinton (50) agar
506 medium supplemented with 5 % w/v fetal calf serum. A 40 µL volume of each isolate
507 suspension (10^9 CFU mL^{-1}) was added to 5 mL phosphate-buffered saline and poured onto
508 the Mueller-Hinton agar plates. After removing excess bacterial suspension by pipetting, the
509 antibiotic discs [oxolinic acid (2 µg), florfenicol (30 µg), sulfamethoxasol/trimethoprim (25
510 µg) and tetracycline (30 µg)] were placed on the plates. The plates were then incubated for 3
511 days at 25°C. After incubation, the inhibition zone around the antibiotic discs was measured.
512 The susceptibility patterns of the selected phage-exposed and no-phage control *F. columnare*
513 isolates to the antibiotics were compared to that of the parent wild-type isolates.
514
515 *Motility/Colony spreading*
516
517 The effect of phage-exposure on bacterial motility was tested by comparing the colony
518 spreading ability of phage-exposed and no-phage control isolates with that of their parent
519 wild-type isolates. After spotting of 5 µL of bacterial suspension (10^9 CFU mL^{-1}) on TYES
520 agar (0.5% agar) plates supplemented with 0.1% baker's yeast and incubation for 3 days at

25ºC, the colony diameter of each isolate was measured. Each isolate was tested in three replicates.

Adhesion and biofilm formation

Changes in adherence or biofilm formation capacities between wild-type, phage-exposed and no-phage control *F. columnare* isolates were studied in flat-bottomed 96-well microtiter plates (Nunclon Δ Surface, Nunc) (51). *F. columnare* cells grown on TYES agar were suspended in autoclaved fresh water (lake Littoistenjärvi) to a concentration of 10^8 CFU mL^{-1} (OD_{520nm}=0.6). For testing of bacterial adherence, a 100 µL volume of the prepared bacterial suspensions were added in triplicate into wells of replicate microtiter plates and incubated statically for 1 h at 25ºC. For testing of biofilm formation, a 100 µL volume of TYES broth was added to wells containing 100 µL of the prepared bacterial suspensions and allowed to incubate for 3 days. Autoclaved fresh water was used as negative control. After incubation, the contents were discarded and the wells were washed three times with sterile 0.5% NaCl to remove non-adherent cells and air dried. The wells were then stained with 0.1% crystal violet solution for 45 min and washed three times by submersion in a container of tap water and air dried. The crystal violet was solubilized with 96% ethanol for 15 min before measuring the absorbance (1 s) at 595 nm (Victor2, Wallac).

Protease activity

Changes in protease activity was examined by spotting 1 µL of bacterial TYES broth suspension (10^8 CFU mL^{-1}) of the wild-type isolates and each phage-exposed and no-phage control isolate on TYES agar (1.5% agar) supplemented with (w/v) elastin (0.1%), gelatin

(3%) and skim milk (5%) (caseinase production). The proteolytic activity of each isolate was observed by the presence of a clear zone surrounding the colony after incubation, and assessed by measuring the clear zone ratio (diameter of clear zone/diameter of the colony) of three replicate samples. In the absence of a clearing zone outside the colony, the clear zone ratio was defined as 1. The measurements were made after 5 (caseinase and gelatinase) or 10 days (elastinase) of incubation at 25ºC.

Virulence

Virulence of phage-exposed and no-phage control *F. columnare* isolates was tested on 1.94 g (average weight) rainbow trout fry and compared to the virulence of wild-type isolates. Fifteen fish per treatment, 20 in control treatment with no bacteria, were exposed individually in 500 mL of bore hole water (25ºC) to cells of single bacterial isolates by constant immersion (5.0 X 10^3 CFU mL^{-1}). Survival of the fish was monitored hourly during 24 h. Morbid fish that did not respond to stimuli were considered dead, removed from the experiment and put down by decapitation. At the end of the experiment, the fish having survived from the infection were put down using 0.008 % Benzocaine. Bacterial cultivations from gills of all the dead fish were made on Shieh agar supplemented with tobramycin (52) to confirm the presence/absence of the bacterium. Cumulative percent mortality and estimated survival time (Kaplan-Meier Survival Analysis), based on observed average survival time of fish after exposure to each isolate, were used as measures of virulence with more virulent isolates having a shorter estimated survival time.

Fish experiment was conducted according to the Finnish Act of Use of Animals for Experimental purposes, under permission ESAVI/8187/2018 granted for Lotta-Riina

571 Sundberg by the National Animal Experiment Board at the Regional State Administrative
572 Agency for Southern Finland.
573
574 *Whole genome sequencing*
575
576 Genomes of the wild-type FCO-F2 and FCO-F9 *F. columnare* and selected (Table 1) 20
577 phage-exposed and four no-phage control isolates were sequenced using Illumina HiSeq
578 platform (Institute of Molecular Medicine Finland). The Illumina data reads of FCO-F9 and
579 its phage-exposed and no-phage control isolates were mapped to a reference genome of *F.*
580 *columnare* isolate B185 (53) using Geneious software version 11.1.5 (Biomatters Ltd.).
581 Genome of the wild-type FCO-F2 isolate was sequenced also using PacBio (BGI, China).
582 PacBio data of FCO-F2 was assembled using > 8kbp reads with Flye (v. 2.7, four iterations)
583 and > 6 kbp with Canu (v. 1.9). These multi-contig assemblies were then combined using
584 Quickmerge (v. 0.3) to produce one 3 221 312 bp contig. This contig was polished with
585 Illumina HiSeq reads using Pilon (v. 1.23), with pre-processing done using Trimmomatic (v.
586 0.39), bowtie2 (2.3.5.1) and Samtools (v. 1.9). The quality of the polished contig was
587 quantified using Busco (v. 4.0.2), which reported 100% completeness of genome against the
588 bacteria_odb10 reference set. The genome was annotated using the NCBI Prokaryotic
589 Genome Annotation Pipeline (PGAP) (54, 55), and used as reference genome for mapping of
590 F2 phage-exposed and no-phage control isolates.
591
592 *Statistical analyses*
593
594 IBM SPSS Statistics version 24 was used for statistical analysis of the data. A one-way
595 analysis of variance (ANOVA) was used to compare means from phenotypic analyses

596 between experimental groups (phage-exposed isolates and no-phage control isolates) and
597 parent wild-type isolates. If needed, lg10, exponential or square root transformations were
598 made for the data to fulfil the homogeneity of variances assumption. If the homogeneity of
599 variances could not be met by transformations, the data were analysed using non-parametric
600 Kruskal-Wallis and Mann-Whitney tests. In case of elastinase and casienase activity, and
601 biofilm formation, the isolates with no activity/biofilm forming capacity were excluded from
602 the ANOVA LSD multiple comparison analyses. Kaplan-Meier Survival Analysis was used
603 for analysis of virulence data.
604
605 *Data availability*
606
607 The whole genome sequences of all the isolates were submitted to GenBank under accession
608 numbers presented in Table 6.
609
610 **Acknowledgements**
611
612 We acknowledge funding from Academy of Finland (grant #314939) and Jane and Aatos
613 Erkko Foundation. This work resulted from the BONUS FLAVOPHAGE project supported
614 by BONUS (Art 185), funded jointly by the EU and Academy of Finland.
615
616 **References**
617
618 1. Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dölz H, Millanao A, Buschmann
619 AH. 2013. Antimicrobial use in aquaculture re-examined: its relevance to

620 antimicrobial resistance and to animal and human health. Environ Microbiol 15:1917–
621 1942.
622 2. Watts JEM, Schreier HJ, Lanska L, Hale MS. 2017. The rising tide of antimicrobial
623 resistance in aquaculture: sources, sinks and solutions. Mar Drugs 15:158.
624 3. Gudding R, Lillehaug A, Evensen Ø (ed). 2014. Fish vaccination. Wiley Blackwell,
625 UK.
626 4. Suomalainen LR, Tiirola MA, Valtonen ET. 2005. Effect of *Pseudomonas* sp. MT5
627 baths on *Flavobacterium columnare* infection of rainbow trout and on microbial
628 diversity on fish skin and gills. Dis Aquat Org 63:61–68.
629 5. Pulkkinen K, Suomalainen LR, Rintamäki-Kinnunen P, Read A, Ebert D, Valtonen
630 ET. 2010. Intensive fish farming and the evolution of pathogen virulence: the case of
631 columnaris disease in Finland. Proc Royal Soc B 277:593-600.
632 6. Schmidt AS, Bruun MS, Dalsgaard I, Pedersen K, Larsen JL. 2000. Occurrence of
633 antimicrobial resistance in fish-pathogenic and environmental bacteria associated with
634 four danish rainbow trout farms. Appl Environ Microbiol 66:4908-4915.
635 7. Hesami S, Parkman J, MacInnes JI, Gray JT, Gyles CL, Lumsden JS. 2010.
636 Antimicrobial susceptibility of *Flavobacterium psychrophilum* Isolates from Ontario.
637 J Aquat Anim Health 22:39–49.
638 8. Molina-Aja A, García-Gasca A, Abreu-Grobois A, Bolán-Mejía C, Roque A, Gomez-
639 Gil B. 2002. Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from
640 cultured penaeid shrimp. FEMS Microbiol Lett 2013:7-12.
641 9. Mohamad N, Amal MNA, Saad MZ, Yasin ISM, Zulkiply NA, Mustafa M,
642 Nasruddin NS. 2019. Virulence-associated genes and antibiotic resistance patterns of
643 *Vibrio* spp. isolated from cultured marine fishes in Malaysia. BMC Vet Res 15:176.

644 10. Declercq AM, Boyen E, Van den Broeck W, Bossier P, Karsi A, Haeseborouck F,
645 Decostere A (2013). Antibiotic susceptibility pattern of *Flavobacterium columnare*
646 isolates collected worldwide from 17 fish species. J Fish Dis 3:45-55.
647 11. Loc-Carrillo C, Abedon ST. 2011. Pros and cons of phage therapy. Bacteriophage
648 1:111-114.
649 12. Nakai T, Sugimoto R, Park KH, Matsuoka S, Mori K, Nishioka T, Maruyama K.
650 1999. Protective effects of bacteriophage on experimental *Lactococcus garviae*
651 infection in yellowtail. Dis Aquat Org 37:33-41.
652 13. Park SC, Nakai T. 2003. Bacteriophage control of *Pseudomonas plecoglossicida*
653 infection in ayu *Plecoglossus altivelis*. Dis Aquat Org 53:33-39.
654 14. Higuera G, Bastías R, Tsersvadze G, Romero J, Espejo RT. 2013. Recently
655 discovered *Vibrio anguillarum* phages can protect against experimentally induced
656 vibriosis in Atlantic salmon, *Salmo salar*. Aquaculture 392-395:128-130.
657 15. Castillo D, Higuera G, Villa M, Middelboe M, Dalsgaard I, Madsen L, Espejo RT.
658 2012. Diversity of *Flavobacterium psychrophilum* and the potential use of its phages
659 for protection against bacterial cold-water disease in salmonids. J Fish Dis 35:193-
660 201.
661 16. Laanto E, Bamford JKH, Ravantti J, Sundberg L-R. 2015. The use of phage FCL-2 as
662 an alternative to chemotherapy against columanris disease in aquaculture. Front
663 Microbiol 6:829.
664 17. Almeida GMF, Laanto E, Ashrafi R, Sundberg LR. 2019. Bacteriophage adherence to
665 mucus mediates preventive protection against pathogenic bacteria. mBio 10:e01984-
666 19.
667 18. Tan D, Svenningsen SL, Middelboe M. 2015. Quorum sensing determines the choice
668 of anti-phage defense strategy in *Vibrio anguillarum*. mBIO 6:00627-15

669 19. Azam AH, Tanji Y. 2019. Bacteriophage-host arm race: an update on the mechanism
670 of phage resistance in bacteria and revenge of the phage with the perspective for
671 phage therapy. Appl Microbiol Biotechnol 103:2121-2131.
672 20. Cohen D, Melamed S, Millman A, Shulman G, Oppenheimer-Shaanan Y, Kacen A,
673 Doron S, Amitai G, Sorek R. 2019. Cyclic GMP–AMP signalling protects bacteria
674 against viral infection. Nature 574:691-695.
675 21. Léon M, Bastias R. 2015. Virulence reduction in bacteriophage resistant bacteria.
676 Front Microbiol 6:343.
677 22. Park SC, Shimamura I, Fukunaga M, Mori KI, Nakai T. 2000. Isolation of
678 bacteriophages specific for a fish pathogen, *Pseudomonas plecoglossicida*, as a
679 candidate for disease control. Appl Environ Microbiol 66:1416-1422.
680 23. Castillo D, Christiansen RH, Dalsgaard I, Madsen L, Middelboe M. 2015.
681 Bacteriophage resistance mechanisms in the fish pathogen *Flavobacterium*
682 *psychrophilum*: Linking genomic mutations to changes in bacterial virulence factors.
683 Appl Environ Microbiol 18:1157-1167.
684 24. Castillo D, Rørbo N, Jørgensen J, Lange J, Tan D, Kalatzis PG, Lo Svenningsen S,
685 Middelboe M. 2019. Phage defence mechanisms and their genomic and phenotypic
686 implications in the fish pathogen *Vibrio Anguillarum*. FEMS Microbiol Ecol
687 95:fiz004
688 25. Kunttu HMT, Suomalainen LR, Jokinen EI, Valtonen ET. 2009. *Flavobacterium*
689 *columnare* colony types: Connection to adhesion and virulence? Microb Pathog 46:
690 21-27.
691 26. Kunttu HMT, Jokinen EI, Sundberg L-R, Valtonen ET. 2011. Virulent and
692 nonvirulent *Flavobacterium columnare* colony morphologies: Characterization of

693 chondroitin AC lyase activity and adhesion to polystyrene. J Appl Microbiol
694 111:1319-1326.
695 27. Laanto E, Bamford JKH, Laakso J, Sundberg LR. 2012. Phage driven loss of
696 virulence in a fish pathogenic bacterium. PLOS ONE 7:e53157.
697 28. Li N, Zhu Y, LaFrentz BR, Evenhuis JP, Hunnicut DW, Conrad RA, Barbier P,
698 Gullstrand GW, Roets JE, Powers JL, Kulkarni SS, Erbes DH, Garcia JC, Nie P,
699 McBride MJ. 2017. The Type IX secretion system is required for virulence of the fish
700 pathogen *Flavobacterium columnare*. Appl Environ Microbiol 83:e017769-17.
701 29. McBride MJ, Nakane D. 2015. Flavobacterium gliding motility and the type IX
702 secretion system. Curr Opin Microbiol 28:72-77.
703 30. Braun TF, Khubbar MK, Saffarini DA, McBride MJ. 2005. *Flavobacterium*
704 *johnsoniae* gliding motility genes identified by mariner mutagenesis. J Bacteriol
705 187:6943-6952.
706 31. Shrivastava A, Johnston JJ, van Baaren JM, McBride MJ. 2013. *Flavobacterium*
707 *johnsoniae* GldK, GldL, GldM, and SprA are required for secretion of the cell surface
708 gliding motility adhesins SprB and RemA. J Bacteriol 195:3201-3212.
709 32. Johnston JJ, Shrivastava A, McBride MJ. 2018. Untangling *Flavobacterium*
710 *johnsoniae* gliding motility and protein secretion. J Bacteriol 200:e00362-17.
711 33. Hunnicutt DW, Kempf MJ, McBride MJ. 2002. Mutations in *Flavobacterium*
712 *johnsoniae gldF* and *gldG* disrupt gliding motility and interfere with membrane
713 localization of GldA. J Bacteriol 184:2370-2378.
714 34. Penttinen R, Hoikkala V, Sundberg LR. 2018. Gliding motility and expression of
715 motility related genes in spreading and non-spreading colonies of *Flavobacterium*
716 *columnare*. Front Microbiol 9:525.

717 35. Labrie S, Samson JE, Moineau S. 2010. Bacteriophage resistance mechanisms. Nat
718 Rev Microbiol 8:317-327.
719 36. Runtuvuori-Salmela A, Kunttu HMT, Laanto E, Almeida GMF, Mäkelä K,
720 Middelboe M, Sundberg LR. (2020). Prevalence of genetically similar
721 *Flavobacterium columnare* phages across aquaculture environments reveals a strong
722 potential for pathogen control. bioRxiv
723 https://biorxiv.org/cgi/content/short/2020.09.23.309583v1
724 37. Porter NT, Hryckowian AJ, Merrill BD, Fuentes JJ, Gardner JO, Glowacki RWP,
725 Singh S, Crawford RD, Snitkin ES, Sonnenburg JL, Martens EC. 2020. Phase-
726 variable capsular polysaccharides modify bacteriophage susceptibility in *Bacteroides*
727 *thetaiotaomicron*. Nat Microbiol 5:1170-1181.
728 38. Laanto E, Hoikkala V, Ravantti J, Sundberg LR. 2017. Long-term coevolution of
729 host-parasite interaction in the natural environment. Nat Commun 8:111.
730 39. Hoikkala V, Ravantti J, Díez-Villaseñor C, Tiirola M, Conrad RA, McBride MJ,
731 Sundberg LR. 2020. Cooperation between CRISPR-Cas types enables adaptation in
732 an RNA-targeting system. bioRxiv https://doi.org/10.1101/2020.02.20.957498
733 40. Kumru S, Tekedar HC, Gulsoy N, Waldbieser GC, Lawrence ML, Karsi A. 2017.
734 Comparative analysis of the *Flavobacterium columnare* Genomovar I and II genomes.
735 Front Microbiol 8:1375.
736 41. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. 2016.
737 Biofilms: an emergent form of bacterial life. Nat Rev 14:563.
738 42. Cai W, De La Fuente L, Arias CR. (2013). Biofilm formation by the fish pathogen
739 *Flavobacterium columnare*: Development and parameters affecting surface
740 attachment. Appl Environ Microbiol 79:5633-5642.

741 43. Decostere A, Haesebrouck F, Turnbull JF, Charlier G. (1999). Influence of water
742 quality and temperature on adhesion of high and low virulence *Flavobacterium*
743 *columnare* strains to isolated gill arches. J Fish Dis 22:1-11.
744 44. Allen RC, Pfrunder-Cardozo KR, Meinel D, Egli A, Hall AR. 2017. Associations
745 among antibiotic and phage resistance phenotypes in natural and clinical *Escherichia*
746 *coli* isolates. mBio 8:e01341-17.
747 45. Zhang QG, Buckling A. 2012. Phages limit the evolution of bacterial antibiotic
748 resistance in experimental microcosms. Evol Appl 5:575-582.
749 46. Song YL, Fryer JL, Rohovec JS. 1988. Comparison of six media for the cultivation of
750 *Flexibacter columnaris*. Fish Pathol 23:91-94.
751 47. Holt RA, Rohovec JS, Fryer JL. 1993. Bacterial cold-water disease, p 3-23. *In* Inglis
752 V, Roberts RJ, Bromage NR (ed), Bacterial Diseases of Fish. Blackwell Scientific
753 Publication Oxford, UK.
754 48. Adams MH. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
755 49. Bauer AW, Kirby WMM, Sherris JC, Turck M. 1966. Antibiotic Susceptibility testing
756 by a standardized single disk method. Am J Clin Pathol 45:493–496
757 50. CLSI. 2006. Methods for antimicrobial disk susceptibility testing of bacteria isolated
758 from aquatic animals; Approved guideline. CLSI document M42-A. Wayne, PA:
759 Clinical and Laboratory Standards Institute.
760 51. Högfors-Rönnholm E, Norrgård J, Wiklund T. 2015. Adhesion of smooth and rough
761 phenotypes of *Flavobacterium psychrophilum* to polystyrene surfaces. J Fish Dis
762 38:429–437.
763 52. Decostere A, Haeseborouck F, Devriese LA. 1997. Shieh medium supplemented with
764 tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter*
765 *columnaris*) from diseased fish. J Clin Microbiol 35:322-324.

766 53. Ravantti JJ, Laanto E, Papponen P, Sundberg LR. 2019. Complete genome sequence
767 of fish pathogen *Flavobacterium columnare* strain B185, originating from Finland.
768 Microbiol Resour Announc 8:e01285-19.
769 54. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L,
770 Lomsadze A, Pruitt KD, Borodovsky M, Ostell J. 2016. NCBI prokaryotic genome
771 annotation pipeline. Nucleic Acids Res 44:6614-24.
772 55. Haft DH, DiCuccio M, Badretdin A, Brover V, Chetvernin V, O'Neill K, Li W,
773 Chitsaz F, Derbyshire MK, Gonzales NR, Gwadz M, Lu F, Marchler GH, Song JS,
774 Thanki N, Yamashita RA, Zheng C, Thibaud-Nissen F, Geer LY, Marchler-Bauer A,
775 Pruitt KD. 2018. RefSeq: an update on prokaryotic genome annotation and curation.
776 Nucleic Acids Res 46(D1):D851-D860.
777

778 **Figures**
779

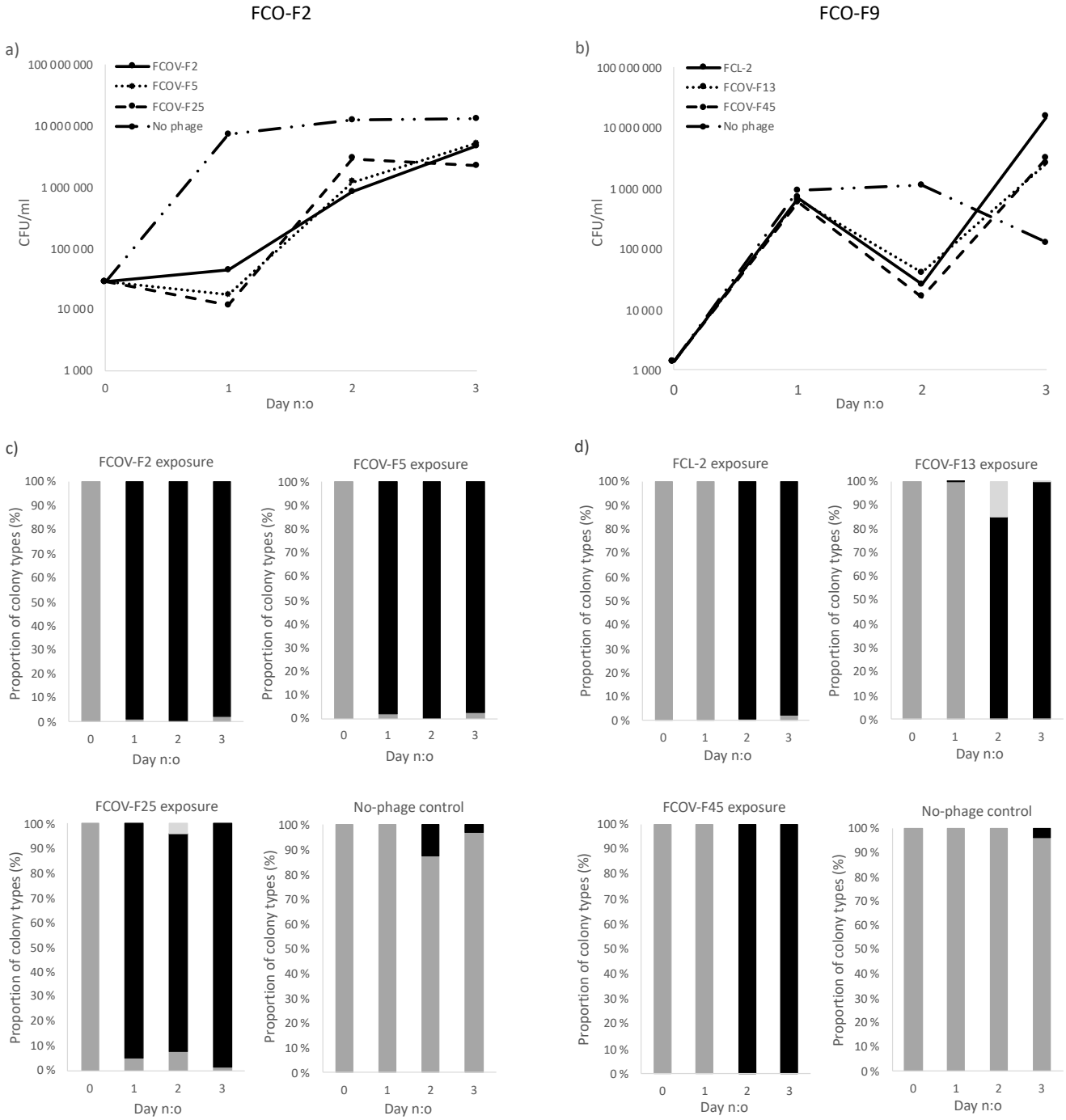

Figure 1. Bacterial growth (a and b), determined as colony forming units mL^{-1}, and proportion (%) of different colony types (c and d) of *Flavobacterium columnare* isolates FCO-F2 (a and c) and FCO-F9 (b and d) during the 3-day exposure to phages FCOV-F2, FCOV-F5, FCOV-F25, FCL-2, FCOV-F13 and FCOV-F45. Dark grey bar: proportion of isolates forming rhizoid colony morphology, black bar: proportion of isolates forming rough colony morphology, light grey bar: proportion of isolates forming soft colony morphology.

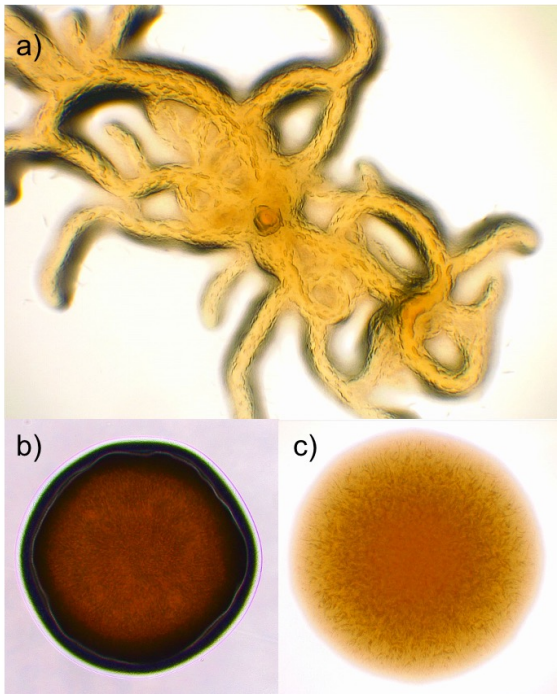

Figure 2. Different colony morphologies formed by *Flavobacterium columnare* on Shieh-agar plates after phage exposure: a) rhizoid, b) rough and c) soft.

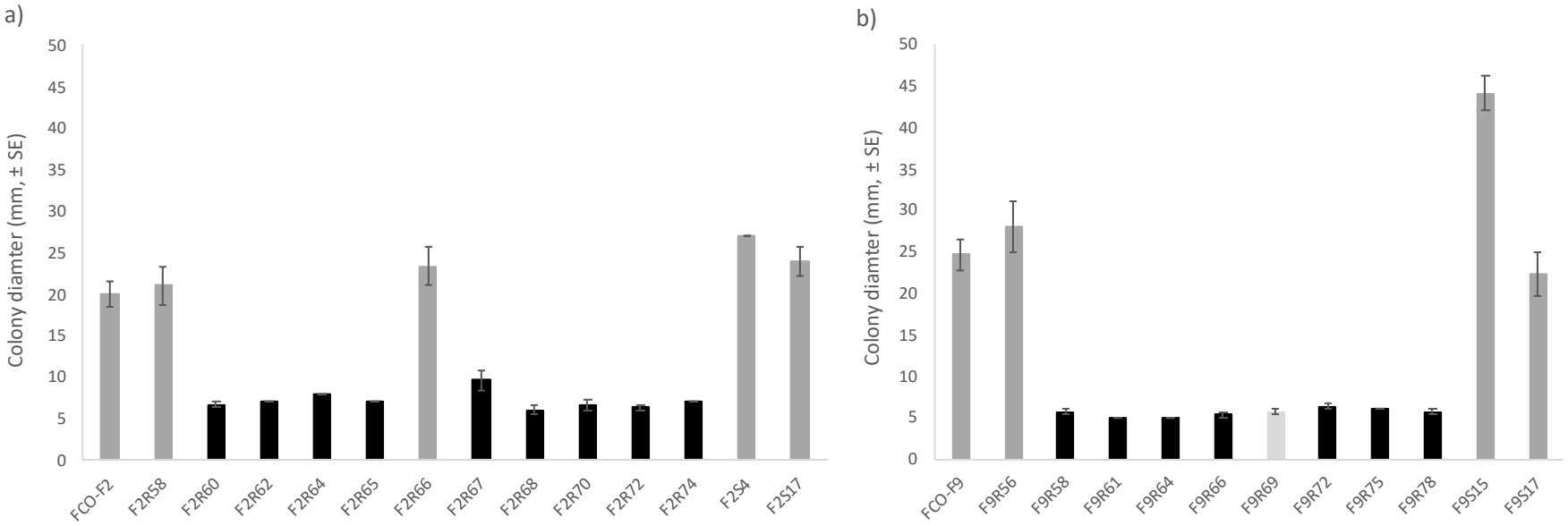

Figure 3. Motility of *Flavobacterium columnare* wild-type a) FCO-F2 and b) FCO-F9 isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates expressed as colony diameter (mm, ±SE) on TYES agar. All the phage-sensitive rhizoid colonies forming isolates (dark grey bar) were significantly more motile than phage-resistant rough (black bar) or soft (light grey bar) morphology isolates (F2-isolates: $P < 0.001$, Oneway ANOVA, LG10 transformation; F9-isolates: $P \leq 0.004$, Mann-Whitney test).

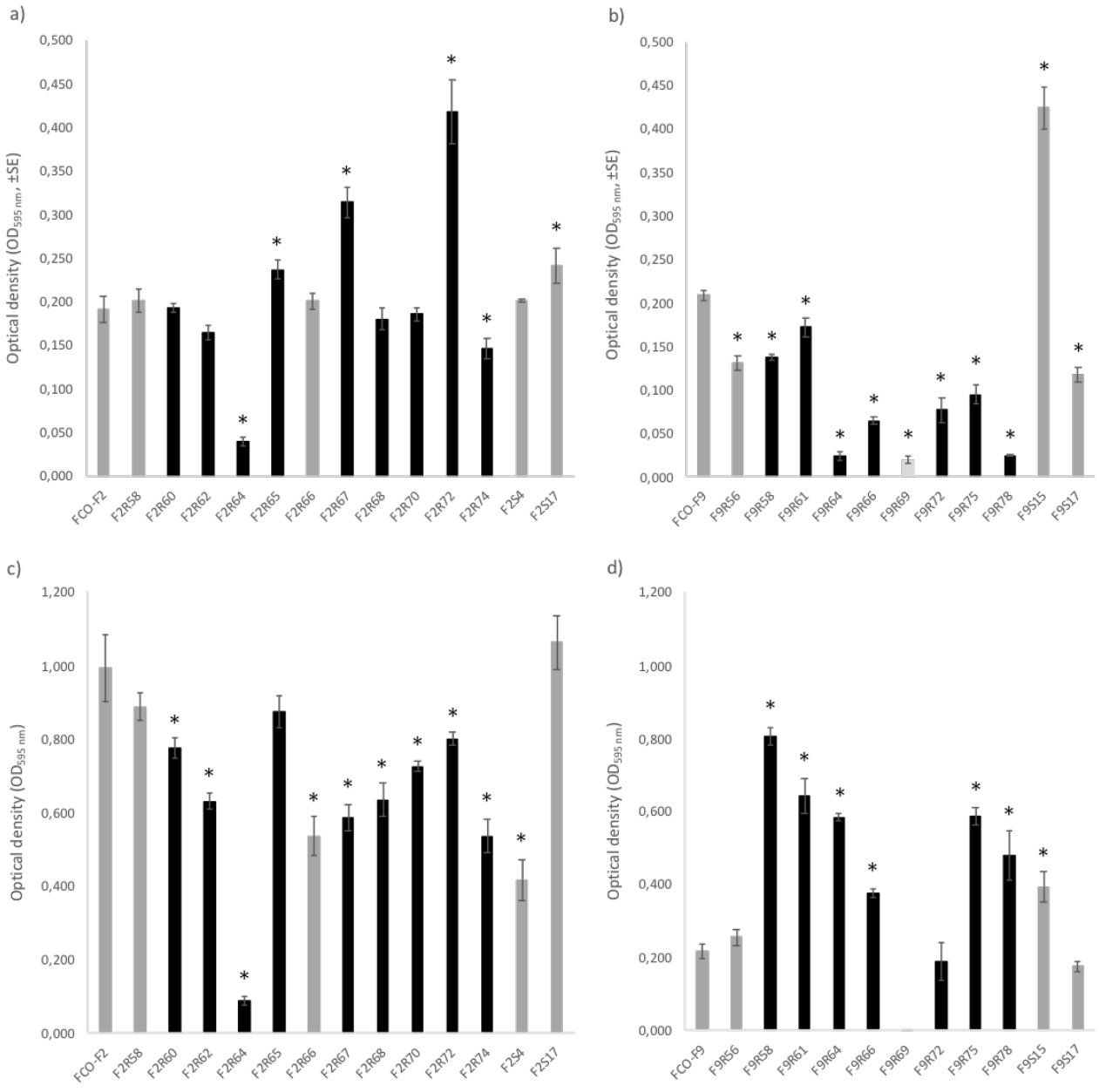

Figure 4. Adherence (a and b) and biofilm forming (c and d) capacity of *Flavobacterium columnare* wild-type FCO-F2 (a and c) and FCO-F9 (b and d) isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates on a polystyrene surface measured as optical density ($OD_{595\ nm}$, ±SE). Asterisks indicate the statistically significant difference ($P < 0.05$) compared to the parent wild-type isolate. F9S69 did not form any biofilm and was thus excluded from the statistical analyses. Dark grey bars: phage-sensitive isolates forming rhizoid colony morphology, black bars: phage-resistant isolates forming rough morphology, light grey bar: phage-resistant isolate forming soft morphology.

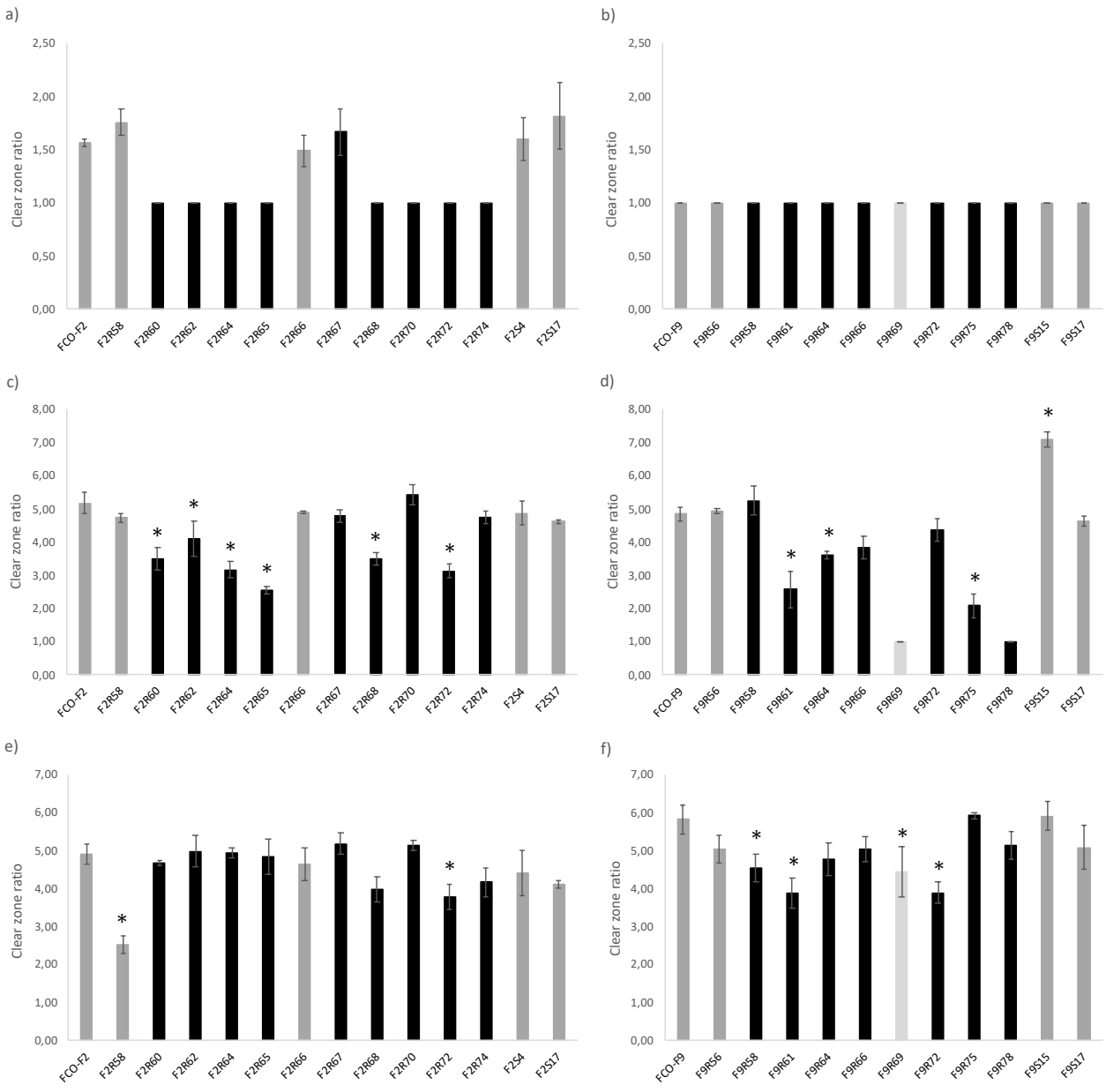

Figure 5. Protease (elastinase: a and b, gelatinase: c and d, and caseinase e and f) activity of the *Flavobacterium columnare* FCO-F2 (a, c and e) and FCO-F9 (b, d and f) isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates. The activity was measured as the clear zone ratio (clear zone diameter/colony diameter, ±SE) on TYES agar supplemented with elastin, gelatin and skim milk (caseinase). The asterisk indicates significant reduction in protease activity ($P < 0.05$) compared to the parent wild-type isolate. A clear zone ratio 1 indicates no protease activity. Isolates with no activity were excluded from the statistical analyses. Dark grey bars: phage-sensitive isolates forming rhizoid colony morphology, black bars: phage-resistant isolates forming rough morphology, light grey bar: phage-resistant isolate forming soft morphology.

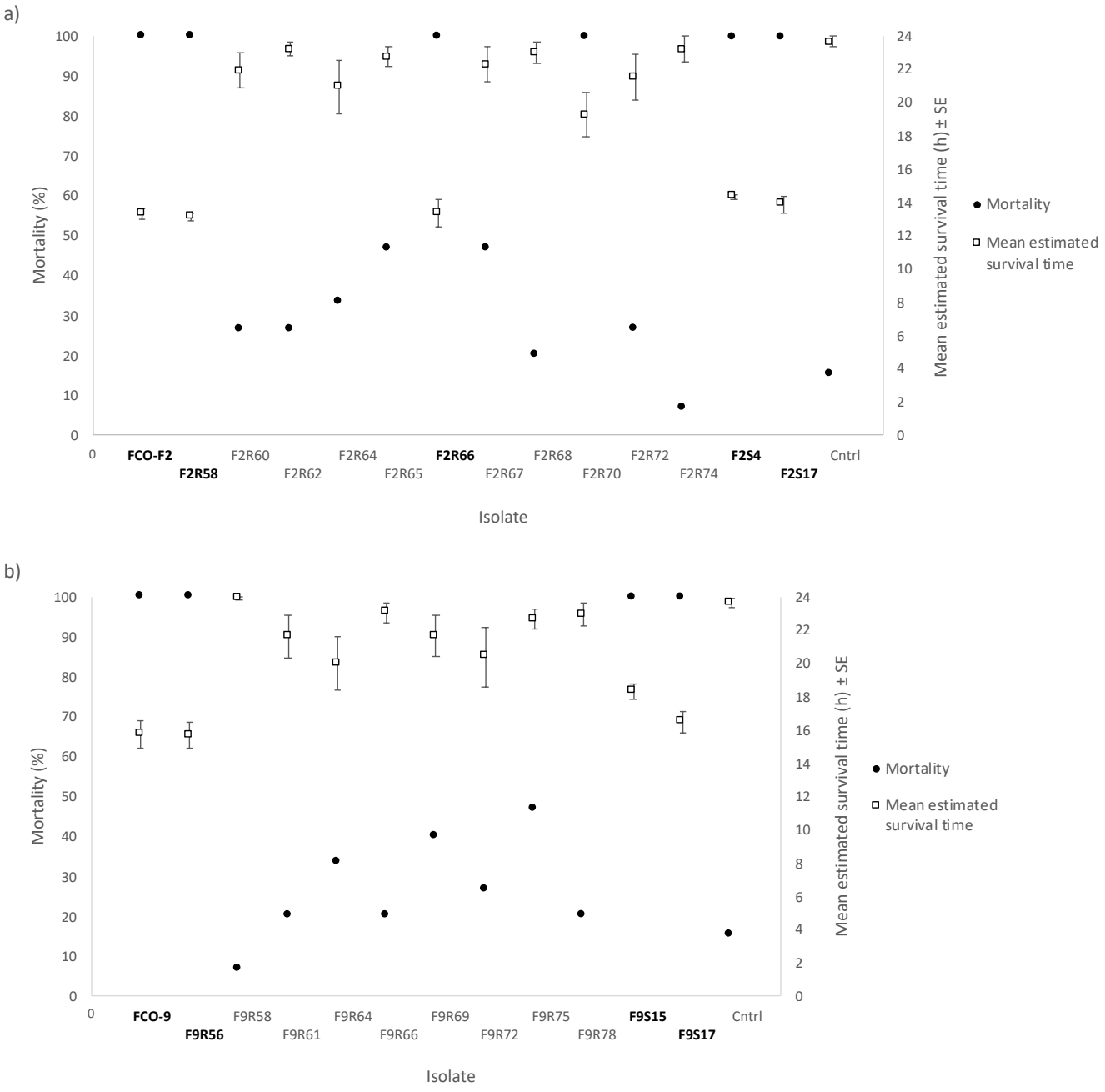

Figure 6. Mortality percent and estimated survival time (± SE) of rainbow trout (*Oncorhynchus mykiss*) during 24-h experimental infection with wild-type *Flavobacterium columnare* FCO-F2 (a) and FCO-F9 (b), and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates. Phage-sensitive rhizoid colonies forming isolates are written bold. Cntrl = control with no bacterial infection.

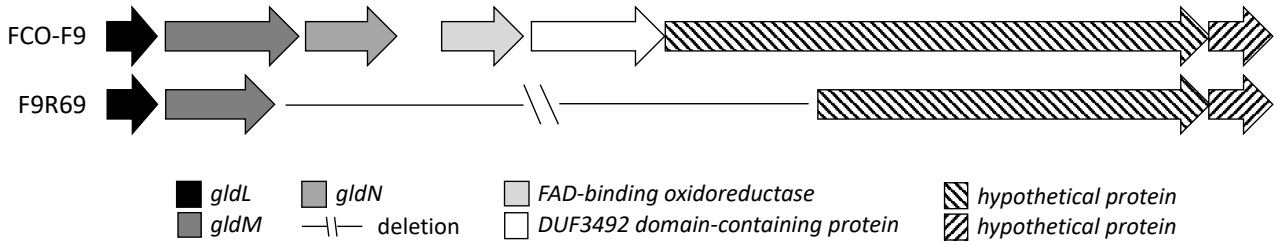

Figure 7. Deletion of genomic region covering 4 701 bp in FCOV-F13 exposed, soft colonies forming, phage-resistant *Flavobacterium columnare* isolate F9R69.

937 **Tables**
938

Table 1. Experimental setup of phage exposure of two phage-sensitive wild-type *Flavobacterium columnare* isolates FCO-F2 (high-virulence, genotype C; exposed for phages FCOV-F2, FCOV-F5 and FCOV-F25) and FCO-F9 (medium-virulence, genotype G; exposed for phages FCL-2, FCOV-F13 and FCOV-F45), and colony morphologies and phage susceptibilities of the 20 phage-exposed (F2R- and F9R-) and 4 no-phage control isolates (F2S- and F9S-) obtained from the exposure cultures. The isolates are shown according to the phage they were exposed to. The susceptibility of the isolates to phages used in exposures: + = sensitive, – = resistant, ± = sensitivity decreased compared to the parent wild-type isolate, i = inhibition of bacterial growth, considered as phage resistance, ND = not determined.

Wild-type isolate	Phage	Phage-exposed isolate	No-phage control isolate	Colony morphology of the isolate	Phage susceptibility of the isolate FCOV-F2	FCOV-F5	FCOV-F25	FCL-2	FCOV-F13	FCO-F45
FCO-F2				rhizoid	+	+	+	ND	ND	ND
	FCOV-F2	F2R58		rhizoid	±	±	±	ND	ND	ND
		F2R60		rough	–	–	–	ND	ND	ND
		F2R62		rough	–	–	–	ND	ND	ND
	FCOV-F5	F2R64		rough	–	–	–	ND	ND	ND
		F2R65		rough	–	–	–	ND	ND	ND
		F2R66		rhizoid	±	±	±	ND	ND	ND
		F2R67		rough	–	–	–	ND	ND	ND
		F2R68		rough	–	–	–	ND	ND	ND
	FCOV-F25	F2R70		rough	i	i	i	ND	ND	ND
		F2R72		rough	–	–	–	ND	ND	ND
		F2R74		rough	–	–	–	ND	ND	ND
	No phage		F2S4	rhizoid	+	+	+	ND	ND	ND
			F2S17	rhizoid	+	+	+	ND	ND	ND
FCO-F9				rhizoid	ND	ND	ND	+	+	+
	FCL-2	F9R56		rhizoid	ND	ND	ND	±	±	±
		F9R58		rough	ND	ND	ND	i	i	i
		F9R61		rough	ND	ND	ND	i	i	i
	FCOV-F13	F9R64		rough	ND	ND	ND	–	–	–
		F9R66		rough	ND	ND	ND	i	i	i
		F9R69		soft	ND	ND	ND	i	i	i
	FCOV-F45	F9R72		rough	ND	ND	ND	i	i	i
		F9R75		rough	ND	ND	ND	i	i	–
		F9R78		rough	ND	ND	ND	–	–	–
	No phage		F9S15	rhizoid	ND	ND	ND	+	+	+
			F9S17	rhizoid	ND	ND	ND	+	+	+

975 **Table 2.** Data on genomes of wild-type *Flavobacterium columnare* strains FCO-F2 and
976 FCO-F9.
977

978 Wild-type isolate	Genetic group	Genome size (bases)	N:o of ORFs	GC %
979 FCO-F2	C	3 221 312	3 280	31.7
980 FCO-F9	G	3 261 403	3 374	31.7

Table 3. Mutations revealed by whole genome sequencing (Illumina) in F2 phage-exposed *Flavobacterium columnare* isolates compared to their wild type (wt) isolate FCO-F2. The isolates are shown according to the phage they were exposed to. CDS = coding sequence, → = change to, Del = deletion, Ins = insertion, aa = amino acid.

(Phage) Phage-exposed isolate	Colony morphology	Gene/CDS	Mutation	Location (base n:o) in wt genome	Outcome
(FCOV-F2)					
F2R58	rhizoid	rlmF	T → A	21 350	No aa change
F2R60	rough	sprA	Ins GT	1 314 323 – 1 314 324	Change in reading frame → stop codon → two truncated proteins
F2R62	rough				
(FCOV-F5)					
F2R64	rough	sprA	Ins G	1 317 523	Change in reading frame → stop codon → two truncated proteins
F2R65	rough	sprA	Ins G	1 317 524	Change in reading frame → stop codon → two truncated proteins
F2R66	rhizoid				
F2R67	rough	gldB	Del T	1 122 801	Truncated/wrong protein
F2R68	rough				
(FCOV-F25)					
F2R70	rough	OmpH family outer membrane protein	Ins G	1 275 242	Change in reading frame → wrong protein
F2R72	rough	gldN	Ins TCTAC	1 013 274 – 1 013 278	Change in reading frame → stop codon → two truncated proteins
F2R74	rough	sprA	Del A	1 313 911	Change in reading frame → stop codon → two truncated proteins

Table 4. Mutations revealed by whole genome sequencing (Illumina) in F9 phage-exposed *Flavobacterium columnare* isolates compared to their wild type (wt) isolate FCO-F9. The isolates are shown according to the phage they were exposed to. CDS = coding sequence, → = change to, Del = deletion, nt = nucleotide, Ins = insertion, aa = amino acid

(Phage) Phage-exposed isolate	Colony morphology	Gene/CDS	Mutation	Location (base n:o) in wt genome	Outcome
(FCL-2)					
F9R56	rhizoid	DegT/DnrJ/EryC1/StrS family aminotransferase	C → T	657 725	Cys → Tyr
		DUF255 domain -containing protein	C → T	2 542 435	Stop codon → truncated protein
F9R58	rough				
F9R61	rough	Cystathionine gamma-synthase	G → A	1 720 857	His → Tyr
(FCOV-F13)					
F9R64	rough	gldM	Del CAA	2 732 551	Del Thr
F9R66	rough	Gliding motility	G → A	1 849 668	Stop codon → truncated protein
F9R69	soft	gldM	Del 255 3’ nt	2 732 457 –	No/truncated protein
		gldN	Del CDS		No protein
		FAD-binding oxidoreductase	Del CDS		No protein
		DUF3492 domain containing protein	Del CDS		No protein
		Hypothetical protein	Del 454 5’ nt	– 2 737 157	No/truncated protein
(FCOV-F45)					
F9R72	rough	gldG	Ins T	3 023 647	Change in reading frame → wrong protein
F9R75	rough	gldN	Ins G	2 733 099	Start and stop codon → two truncated proteins
F9R78	rough	gldM	Ins A	2 731 567	Change in reading frame → stop codon → truncated protein

Table 5. *Flavobacterium columnare* isolates and phages used in this study. Bacteria and phages were isolated from Finnish fish farms. *F. columnare* isolates have previously been categorized into genetic groups by restriction fragment length polymorphism analysis of internal transcribed spacer region between 16S and 23S rRNA genes (This article was submitted to an online preprint archive [36]).

Bacterium isolate	Genetic group of the bacterium	Phage isolate	Genetic group of the phage isolation host	Farm number	Isolation year
FCO-F2	C			1	2017
FCO-F9	G			2	2017
		FCOV-F2	C	1	2017
		FCOV-F5	C	3	2017
		FCOV-F25	C	1	2017
		FCL-2	G	2	2009
		FCOV-F13	G	1	2017
		FCOV-F45	G	2	2017

Table 6. Accession numbers of whole genome sequences of wild-type *Flavobacterium columnare* isolates FCO-F2 and FCO-F9 and their phage-exposed (F2R- and F9R) and no-phage control (F2S- and F9S-) isolates submitted to GenBank.

Isolate	Accession number
FCO-F2	CPO51861
F2R58	CP054506
F2R60	CP054505
F2R62	CP054504
F2R64	CP054503
F2R65	CP054502
F2R66	CP054501
F2R67	CP054500
F2R68	CP054499
F2R70	CP054498
F2R72	CP054497
F2R74	CP054496
F2S4	CP054495
F2S17	CP054494
FCO-F9	CP054518
F9R56	CP054517
F9R58	CP054516
F9R61	CP054515
F9R64	CP054514
F9R66	CP054513
F9R69	CP054512
F9R72	CP054511
F9R75	CP054510
F9R78	CP054509
F9S15	CP054508
F9S17	CP054507