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

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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.

46 **Importance**

47

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

60

61 **Introduction**

62

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

71 with no potent immunization method available (3). This applies especially to infections of fish
72 fry, where efficiency of vaccination is poor due to lack of development of fish secondary
73 immunity at the early life stage.
74
75 One of these diseases affecting fry is caused by the fish pathogenic bacterium *Flavobacterium*
76 *columnare*, the infectious agent of columnaris disease. Columnaris infections cause extensive
77 losses in farmed salmonid fry and juveniles, populations of different catfish species and ayu
78 (*Plecoglossus altivelis*) around the world in water temperatures above 18 °C. The only effective
79 curing method is antibiotic treatment. However, infections often occur repeatedly and may
80 cause up to 100% mortality in rainbow trout fry populations if not treated, thus causing major
81 economic losses to the industry (4, 5). In addition, elevated water temperatures due to warmer
82 summers in the recent years are suggested to enhance virulence development in *F. columnar*e
83 (5). Although antibiotic resistance in this bacterium is not yet as severe problem as in related
84 pathogens, e.g. *Flavobacterium psychrophilum* (6, 7) or *Vibrio* species (8, 9), strains that have
85 acquired resistance towards commonly used antibiotics already exist (10).
86
87 Bacteriophages (phages) are viruses that specifically infect their host bacteria, without harming
88 the surrounding microbial community (reviewed in 11). Among the alternatives to traditional
89 antibiotics, phage therapy, i.e. the use of phages against bacterial infections, has demonstrated
90 a strong potential for controlling disease outbreaks in aquaculture (e.g. 12-14). Promising
91 results have been gained also in phage therapy trials of Flavobacterial infections. In a study by
92 Castillo et al. (15), phage treatment reduced the mortality of *F. psychrophilum*-infected
93 Atlantic salmon (*Salmo salar*) by 60 % and rainbow trout (*Oncorhynchus mykiss*) by 67 %. In
94 studies with columnaris infections, mortality of zebra fish (*Danio rerio*) and rainbow trout were
95 reduced by 100 % and nearly 42 %, respectively, in the presence of phages (16). In addition,

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

121 Understanding the mechanisms and consequences of phage resistance in the target bacteria is
122 central for development of successful phage therapy. Thus, in this study, we exposed two *F.*
123 *columnare* isolates (FCO-F2 and FCO-F9) separately to three different phages, and studied
124 infection dynamics, bacterial viability and colony morphology, and isolated phage-resistant
125 bacteria. Twenty-four phage-exposed and no-phage control isolates were further characterized
126 for their phage resistance, antibiotic susceptibility, motility, adhesion and biofilm formation on
127 polystyrene surface, protease (elastinase, gelatinase and caseinase) activity, virulence on
128 rainbow trout fry, and whole genome sequence. Our results show, that if phage resistance in *F.*
129 *columnare* is gained via surface modification leading to morphotype change, virulence
130 decreases. However, if the colony morphology remains rhizoid, the isolates remain highly
131 virulent with reduced sensitivity to phage compared to the ancestral wild-type strain.
132
133 **Results**
134
135 *Isolates from phage-exposures: growth, colony morphology and phage resistance*
136
137 In all phage-exposure cultures of FCO-F2, there was a strong initial phage control of the host
138 population during the first day in all the phage-exposed cultures compared with control culture
139 without phages (Figure 1a). After this, the bacterial density started to recover. The phage-free
140 cultures grew exponentially during the first day, after which they reached a plateau phase.
141 Along with the population decline on day 1, bacterial colony morphotype changed from
142 ancestral rhizoid to rough (Figure 2). From day 1 onwards, more than 88% of the colonies
143 formed by phage-exposed bacterial isolates were rough, the amount reaching at least 97% at
144 the end of the experiment (Figure 1c). In addition, in FCOV-F25 exposure, few soft colonies

145 were observed on day 2 (Figure 2), and in no-phage control cultures, some rough colonies
146 appeared among the prevailing rhizoid ones.
147
148 FCO-F9 showed slightly different growth dynamics. The bacterial population size increased
149 exponentially during the first day in all cultures (Figure 1b), but decreased drastically on day
150 2 in response to phage exposure, and then reached exponential growth again. The phage-free
151 cultures reached a plateau phase on day 2, after which the amount of culturable bacteria
152 decreased. From the day 2 population crash and onwards, more than 85% of the colonies
153 formed by phage-exposed bacteria had rough morphology (Figure 1d). At the end of the
154 experiment, more than 98% of the colonies where rough. In FCOV-F13 exposure, a few rough
155 colonies were observed already on day1 and some soft colonies on days 2 and 3. In no-phage
156 control cultures, some (4 %) rough colonies appeared among the rhizoid ones on day 3.
157
158 Out of 189 colonies collected from phage exposures, 20 phage-exposed and 4 no-phage control
159 isolates were characterized further (Table 2). Of these isolates, the no-phage control isolates
160 all formed rhizoid colonies similar to their wild-type parent, phage-sensitive isolates FCO-F2
161 and FCO-F9. Most of the phage-exposed isolates were of rough colony morphology, but
162 F2R58, F2R66 and F9R56 had rhizoid, and F9R69 soft colony morphology.
163
164 All the phage-exposed rough isolates were resistant to all the phages used to infect the ancestor
165 wild-type bacteria (Table 2). In addition, in some cases, phage caused inhibition of bacterial
166 growth, considered as phage resistance because no clear plaques due to phage infection were
167 detected. The rhizoid phage-exposed isolates turned out to be partly phage-resistant with a 5.5
168 X 10^5 to 11 X 10^5-fold reduction in phage susceptibility compared to the wild type isolates,

169 depending on the specific phage (results not shown). Throughout this paper, these isolates with
170 decreased phage sensitivity are grouped together with the phage-sensitive isolates.
171
172 *Antibiotic susceptibility*
173
174 All isolates showed antibiotic susceptibility patterns similar to the parent wild-type isolates,
175 and no notable differences were observed (Figure S1 and Table S1).
176
177 *Motility, adhesion and biofilm formation*
178
179 Phage-sensitive bacteria forming rhizoid colonies were significantly more motile (determined
180 as colony spreading) than phage-resistant rough or soft morphotypes, irrespective of isolation
181 history (F2-isolates: $P < 0.001$, Oneway ANOVA, LG10 transformation; F9-isolates: $P \leq$
182 0.004, Mann-Whitney test) (Figure 3).
183
184 Compared to the parent wild-type FCO-F2 isolate, there was a large variability on the adhesion
185 capacity of individual phage-resistant F2-isolates (Figure 4a). Phage susceptibility (rhizoid vs.
186 rough colony type) or phage used in the co-culture experiment did not influence bacterial
187 adhesion capacity ($P = 0.3$: Mann-Whitney test and $P = 0.564$: Kruskal-Wallis test,
188 respectively).
189
190 Most of the individual phage-exposed and no-phage control F2-isolates had significantly lower
191 biofilm forming capacity than in parent wild-type FCO-F2 ($P \leq 0.017$: Oneway ANOVA, LDS
192 multiple comparisons, square root transformation) (Figure 4c). Still, there was no statistical

193 difference in biofilm formation between phage-sensitive rhizoid and resistant rough
194 morphology F2-isolates ($P = 0.062$: Oneway ANOVA).
195
196 Again, the bacterial strain F9 behaved differently compared to F2. In contrast to the phage-
197 resistant F2-isolates, the phage-resistant rough and soft morphology F9-isolates had
198 significantly lower adherence than sensitive rhizoid isolates ($P < 0.001$: Oneway ANOVA,
199 LDS multiple comparisons, square root transformation) (Figure 4b). In addition, isolates
200 exposed to phages isolated in 2017, FCOV-F13 and FCOV-F45, had significantly lower
201 adhesion capacity than in isolates exposed to FCL-2 isolated in 2009 ($P < 0.001$: Mann-
202 Whitney test). This may indicate phage FCL-2 uses different phage receptor (see later).
203
204 In contrast to adhesion ability, biofilm forming capacity of the most of the individual phage-
205 exposed and no-phage control F9-isolates was significantly higher compared to wild-type
206 parent isolate ($P \leq 0.004$: Oneway ANOVA, LDS multiple comparisons) (Figure 4d). F9R69
207 with soft colony morphology did not form any biofilm and thus excluded from the multiple
208 comparisons. Phage-resistant rough F9-isolates had significantly higher biofilm forming
209 capacity than sensitive rhizoid morphotypes ($P < 0.001$: Oneway ANOVA, square root
210 transformation).
211
212 *Protease activity: elastinase, gelatinase and caseinase*
213
214 Elastinase activity was detected in the wild-type, and all the phage-sensitive rhizoid FCO-F2
215 isolates and one resistant rough F2-isolate (clear zone ratio > 1), whereas all remaining
216 resistant, rough morphology isolates, had completely lost the ability to degrade elastin (Figure
217 5a). There were no differences in elastinase activity between the elastinase positive isolates (*P*

218 = 0.843: Oneway ANOVA). Elastinase activity was not detected in any of the F9-isolates (clear
219 zone ratio = 1) (Figure 5b).
220
221 There were variations in gelatinase activity between individual F2- and F9-isolates (Oneway
222 ANOVA, LDS multiple comparisons) (Figure 5c and d). However, among both F2- and F9-
223 isolates, gelatinase activity of phage-resistant rough morphotypes was lower than that of
224 sensitive rhizoid morphotypes (F2-isolates: $P = 0.018$, Oneway ANOVA, exponential
225 transformation; F9-isolates: $P < 0.001$, Oneway ANOVA). Two of the phage-exposed F9-
226 isolates (F9R69 and F9R78) did not have any gelatinase activity and were thus excluded from
227 the multiple comparisons
228
229 Less variation in caseinase activity between individual isolates was observed (Oneway
230 ANOVA, LDS multiple comparisons) (Figure 5e and f), and phage-sensitive rhizoid and
231 resistant rough F2-isolates did not differ from each other ($P = 0.058$: Oneway ANOVA. On the
232 other hand, caseinase activity of phage-resistant rough and soft F9-isolates was lower than that
233 of sensitive rhizoid isolates ($P = 0.007$: Oneway ANOVA).
234
235 *Virulence*
236
237 Rainbow trout fry were exposed to wild-type, phage-exposed and no-phage control isolates,
238 and all of them caused mortality during 24 h (Figure 6). The phage-sensitive rhizoid
239 morphotypes were most virulent, causing 100 % mortality, whereas resistant rough and soft
240 morphotypes were less virulent, causing 46.7 % mortality at highest (except for phage-resistant
241 rough morphotype F2R70, which caused 100 % mortality). Mortality of control fish was 15 %,
242 but no bacterial growth was observed from these fish. However, *F. columnare* growth was

243 observed from all the fish exposed to bacteria. Colony morphotype of the bacterial isolates did
244 not change during the infection.
245
246 When comparing the data according to the phage susceptibility and thus colony morphology,
247 cumulative mortality of fish infected with phage-sensitive rhizoid morphotypes, irrespective of
248 if they were wild-type, phage-exposed or no-phage control isolates, was significantly higher
249 than mortality caused by phage-resistant rough or soft morphotypes among both F2 and F9
250 isolates ($P < 0.001$, Kaplan-Meier Survival Analysis). Also, the estimated survival time
251 (Kaplan-Meier Survival Analysis) was shortest in fish infected with sensitive rhizoid isolates
252 (Figure 6). In case of F2-isolates, mortality caused by phage-resistant rough isolates was also
253 significantly higher than mortality of control fish, but mortality caused by resistant rough and
254 soft F9-isolates did not differ from each other or from the control fish mortality. Mortality
255 caused by rhizoid phage-sensitive F2 isolates started to peak at 12 hours post infection (p.i.)
256 and in F9 at 16 hours p.i. ($P < 0.001$, Kaplan-Meier Survival Analysis), but between rough
257 phage-resistant F2 and F9 isolates the mortality patterns were more similar starting to increase
258 slowly at 2-3 hours p.i. ($P = 0.217$, Kaplan-Meier Survival Analysis). However, there were
259 differences in cumulative mortalities caused by individual isolates in each morphology group
260 (Data set S1).
261
262 *Whole genome sequencing*
263
264 Genome data of wild-type *F. columnare* isolates FCO-F2 and FCO-F9 is presented in Table 4.
265
266 Genomic comparisons between F2 wild type and phage-exposed isolates revealed a limited
267 number of genomic changes. In seven out of 11 isolates, single mutation leading to formation

268 of wrong or truncated proteins was observed in the phage-resistant mutants (Table 5). Notably,
269 the majority of the mutations were located in genes coding for gliding motility proteins *gldB*
270 (F2R67), *gldN* (F2R72) and *sprA* (F2R60, F2R64, F2R65, F2R74). Isolate F2R70 had one
271 nucleotide insertion in OmpH family outer membrane protein coding gene. Three isolates
272 (F2R62, F2R66, F2R68) did not show any genomic changes relative to the wild type. In isolate
273 F2R58 with decreased phage sensitivity, one nucleotide change in *rlmF* gene (coding for rRNA
274 large subunit methyltransferase F) did not lead to amino acid change. No mutations were
275 observed in the no-phage control isolates. At certain points of ribosomal RNA operons in all
276 phage-exposed and no-phage control isolates, and also in a 736 221 bp sequence (hypothetical
277 protein coding sequence in wild-type FCO-F2 genome used as a reference) in phage-exposed
278 isolates F2R66 and F2R68, there was a poor coverage of reads leading to unclear sequences,
279 which prevented detection of possible mutations in this region.
280
281 In F9 phage-exposed isolates, one or two mutations per isolate in all the other isolates, except
282 for F9R58, were observed (Table 6). Mutations in isolates exposed to FCOV-F45 had
283 insertions whereas FCOV-F13 exposed isolates had deletions or single nucleotide chances in
284 genes coding for gliding motility proteins *gldG* (F9R72), *gldM* (F9R64, F9R69, F9R78) and
285 *gldN* (F9R69, F9R75), leading to formation of wrong or truncated proteins. Interestingly, in
286 the isolate F9R69 (exposed to FCOV-F13) with a soft colony type, a deletion of genomic region
287 of 4 701 bp was observed, spanning over gliding motility genes *gldM* and *gldN,* and sequences
288 coding for FAD-binding oxidoreductase, DUF3492 domain-containing protein and a
289 hypothetical protein (Figure 7).
290
291 On the contrary, no mutations in gliding motility genes were observed in F9 isolates exposed
292 to FCL-2, but instead, two of these isolates had one nucleotide change in

293 DegT/DnrJ/EryC1/StrS family aminotransferase and DUF255-domain containing protein
294 (F9R56), and cystathionine gamma-synthase (F9R61) coding genes, leading to either one
295 amino acid change or truncated protein. No mutations were observed in no-phage control
296 isolates. Around 2 000 620 bp (hypothetical protein coding sequence in B185 genome used as
297 a reference), there was a poor coverage of reads leading to unclear sequence in both wild type
298 FCO-F9, phage-exposed and no-phage control isolates, which prevented detection of possible
299 mutations in this region.
300
301 **Discussion**
302
303 Phage therapy is seen as an attractive option to treat and prevent bacterial diseases, but the
304 development of phage resistance in target bacteria is considered as one of the main problems
305 related to the use of phages. Our results describe the selection for phage resistance in two
306 different *F. columnare* isolates upon exposure to six specific phages. We show that phage
307 resistance is associated with reduction in virulence and virulence-related phenotypic changes
308 in the bacterium. Our genetic data indicate that in most cases phage resistance is caused by
309 surface modifications, often related to the type IX secretion system connected to flavobacterial
310 gliding motility machinery. Mutations in the genes coding for an outer membrane protein or
311 genes related to gliding motility seem to be phage specific and likely prevent phage attachment,
312 possibly in a phage specific manner, and lead to morphology change and loss of virulence.
313
314 In the present study, phage-exposure caused significant changes in bacterial phenotypic
315 characteristics (motility, adhesion, protein secretion and virulence - details below) leading to
316 phage resistance. In most isolates, these changes could be linked to changes in gliding
317 motility-related genes. Flavobacteria show gliding motility on surfaces (29), and mutations in

318 any of the genes coding for gliding motility machinery proteins have been shown to lead to
319 loss of motility (e.g. 30, 31). Gliding is also connected to virulence, since part of the gliding
320 motility machinery (GldK, GldL, GldM, GldN, PorV, SprA, Spr E, SprF and SprT) is used as
321 a type IX secretion system found in Bacteroidetes (28, 32). Indeed, phage resistance due to
322 loss of motility has been linked with decreased virulence in *F. columnare* also previously
323 (27), and *F. columnare gldN* mutants have been shown to exhibit both decreased proteolytic
324 and chondroitinase activity, and virulence on rainbow trout (28). Similarly, phage resistance
325 was associated with loss of motility and mutations in genes related to cell surface properties
326 and gliding motility in *F. psychrophilum* (23) and in *F. johnsoniae* (31, 33). Together, the
327 results suggest that the type IX secretion system is a key target for infection by a wide range
328 of phages and across the Flavobacterium genus, and that mutations leading to morphology
329 changes and loss of motility is a general response to phage exposure in this bacterial group.
330
331 Exposure to a specific phage led to different mutations in gliding motility genes in different
332 *F. columnare* isolates, as also seen in phage-resistant *F. psychrophilum* (23), indicating that
333 several genes are involved in phage attachment and infection of *F. columnare* phages.
334 Furthermore, genomic analysis of one soft colony isolate revealed a large deletion (4 701 bp),
335 spanning over two gliding motility genes. However, although all rough colony forming
336 isolates were phage-resistant, not all these isolates (F2R62, F2R66, F2R68 and F9R58) had
337 mutations in genes coding for proteins related to gliding motility, or elsewhere in their
338 genome. This may indicate that development of phage resistance and colony morphology
339 change are also influenced by gene expression or epigenetic modifications, leading to
340 variation in colony morphology, as suggested previously (34). For example, in *Bordetella*
341 spp, phage resistance is regulated via phase variation in virulence related factors, such as
342 some adhesins, toxins and type III secretion system (reviewed in 35). Interestingly, isolates

343 exposed to FCL-2 did not have mutations in gliding motility related genes, suggesting that
344 FCL-2 uses other receptors for infection of *F. columnare* than the other phages. FCL-2
345 differs genetically from other phages infecting genetic group G bacteria (This article was
346 submitted to an online preprint archive [36]), supporting this suggestion.
347
348 Generally, point mutations and changes in receptor expression enable a rapid and efficient
349 response of bacterial populations to phage exposure. However, the large phenotypic costs of
350 mutational derived phage resistance observed in *F. columnare* in this study suggest that these
351 mutations may be dynamic and most probably also rapidly reverting back to the sensitive
352 form in *F. columnare*. Indeed, reversion of both phage-driven and spontaneously formed
353 rough colony types back to rhizoid has been observed to happen in *F. columnare* subcultures
354 (27). Various mechanisms to regain phage resistance have been found also in fish pathogenic
355 *F. psychrophilum* (23) and *V. anguillarum* (24), in which a rapid reversion back to phage-
356 sensitive phenotype has been shown to occur. This sort of dynamics in phage resistance has
357 also been observed in a human symbiont *Bacteroides thetaiotaomicron* (37), suggesting that
358 the phenomenon may be common among wide variety of bacteria.
359
360 Phage-exposed *F. columnare* isolates F2R56, F2R66 and F9R58 did not respond to phage
361 infection with surface modifications, but maintained their original rhizoid colony morphotype
362 and high virulence. These rhizoid isolates were not completely resistant to phage, although
363 phage infection efficiency dropped markedly (up to a million-fold decrease), suggesting some
364 other mechanism for reducing infection efficiency. *F. columnare* has two functional CRISPR
365 systems, which have been shown to adapt under phage exposure at fish farms (38). However,
366 we did not observe additional CRISPR spacers in whole genome sequencing. The same was
367 observed in phage exposed *F. psychrophilum* isolates in which no differences to the wild-

368 type strain's CRISPR composition were found (23). In our experience, CRISPR adaptation in
369 *F. columnare* requires different experimental set-up with longer co-culture time in low
370 nutrient medium, followed by enrichment in high-nutrient medium (This article was
371 submitted to an online preprint archive [39]). Thus, the decreased phage sensitivity of rhizoid
372 phage exposed isolates most probably is a consequence of yet unknown functions which need
373 to be studied in the future.
374
375 In addition to type IX secretion system, also type I and VI secretion systems are known to
376 function in *F. columnare* (40). Possible secretion of virulence related factors through type I
377 and VI secretion systems in *F. columnare* could be one of the reasons why also rough phage-
378 resistant isolates caused some mortality in fish, and explain their gelatinase and caseinase
379 activity despite morphology change. It has also been shown recently, that virulence of *F.*
380 *columnare* increases in the mucus and with increasing mucin concentration (17). As the mucus-
381 covered fish surface is the main infection route of *F. columnare*, it is probable that some *F.*
382 *columnare* virulence factors, such as proteinase activity, are expressed differently in growth
383 media compared to the *in vivo* infection situation. This possible differential expression could
384 also explain the mortality caused by phage-resistant rough isolates.
385
386 The ability to adhere and form biofilm has a major role in bacterial infections and in colonizing
387 niches (41). In *F. columnare*, adhesion and biofilm forming capacity may have a central role
388 in their persistence in the farming environment (e.g. tanks and water systems) (42), but also in
389 establishing the first steps of infection on the fish surfaces (43). Our results indicate that *F.*
390 *columnare* strains differ in their adherence and biofilm forming characteristics. Whereas phage
391 exposure had no clear effect on the adhesion capacity of the F2-isolates, phage resistance led
392 to decrease in biofilm forming capacity in most of the individual phage-resistant F2-isolates.

393 This is in agreement with the systematic reduction in biofilm forming properties of phage-
394 resistant *F. psychrophilum* relative to the wild type (23). Adhesion capacity of F9 phage-
395 resistant isolates, on the other hand, was significantly lower compared to the wild-type parent
396 isolate, but rough phage-resistant F9-isolates had significantly higher biofilm forming capacity
397 compared to rhizoid sensitive isolates. These results partly differ from what we have found
398 earlier (25, 26), most likely because in the previous studies the rough colonies were formed
399 spontaneously, without phage exposure. Indeed, morphology of spontaneously formed rough
400 colonies and these morphotypes' ability to move when cultured in low-nutrient media differ
401 from rough morphotypes formed under phage exposure (27). However, together our results
402 indicate, that since *F. columnare* phages are genetically group-specific, they might be using
403 different receptors, which, in turn, causes differences in bacterial resistance mechanisms
404 between genetic groups.
405
406 *F. columnare* infections are routinely treated by antibiotics at fish farms. In this study, phage
407 resistance did not affect the antibiotic susceptibility of any of the isolates studied. Lack of
408 association between development of antibiotic resistance and bacteriophage resistance has also
409 been shown e.g. in *Escherichia coli* (44). Based on our results, phage resistance does not
410 increase a risk of antibiotic resistance development, and thus, phage-therapy given as a cure or
411 prophylactic treatment at fish farms most probably does not rule out the possible concomitant
412 use of antibiotics as therapeutic agents against columnaris infections. Indeed, it was shown by
413 using *P. fluorescence* as a model bacterium, that applying phages together with antibiotic
414 treatments may inhibit the evolution of antibiotic resistance in pathogenic bacteria (45).
415
416 To summarize, our results show, that even though *F. columnare* rapidly develops phage
417 resistance under phage exposure, the arise of phage resistance does not pose a high risk for a

418 development of phage therapy against columnaris infections in rainbow trout. This is because
419 phage resistance leads to decrease in bacterial virulence, adherence to surfaces and protease
420 secretion. Based on our results with experiments with two genetically different wild-type
421 bacterial isolates, development and regulation of phage resistance in *F. columnare* is a
422 multifactorial process, partly affected by formation of mutations mainly in gliding motility and
423 type IX secretion system related genes, and partly by other defence mechanisms against
424 phages, the function of which needs to be studied in the future.
425
426 **Materials and methods**
427
428 *Bacterial and phage isolates*
429
430 Bacteria and phages used in this study were isolated from water samples collected from fish
431 farms during columnaris outbreaks (This article was submitted to an online preprint archive
432 [36]) (Table 1). Bacteria were confirmed as *F. columnare* by restriction fragment length
433 polymorphism (RFLP) analysis of 16S rRNA gene and classified into genetic groups by RFLP
434 of 16S-23S internal transcribed spacer (ITS) region (This article was submitted to an online
435 preprint archive [36]). All the six phages belong to the *Myoviridae* family and have been
436 characterised with respect to host range and genomic composition (This article was submitted
437 to an online preprint archive [36]).
438
439 *Bacterial cultures and phage lysates*
440
441 For phage exposure and virulence test, *F. columnare* isolates were inoculated from
442 cryopreserved (–80°C) stocks in modified Shieh-medium (46) and grown for 48 h at 25°C with

443 120 rpm agitation. After this, subcultures were made in modified Shieh-medium and grown for
444 24 h at 25°C with 120 rpm agitation. The optical density (OD) of the bacterial broth
445 suspensions was measured spectrophotometrically at 595 nm and adjusted to 5 X 10^5 colony
446 forming units (CFU) mL^{-1} for phage exposures and 5 X 10^6 CFU mL^{-1} for virulence experiment
447 (based on previously determined OD/CFU relationship). For other test, *F. columnare* isolates
448 were cultured in TYES broth (47), washed in TYES broth by centrifugation at 5310 X *g* for 15
449 min at 4°C, and cultures spectrophotometrically adjusted to OD 0.6 at 520 nm (approximately
450 10^8 CFU mL^{-1}).
451
452 Phage lysates were produced using "double layer agar" -method (48) as follows: Three mL of
453 melted (47°C) top agar (0.5%) including 300 µL of 24-hour subculture of the host bacterium
454 and 100 µL of phage (tenfold dilutions in Shieh medium) was poured on Shieh agar and grown
455 for 48 h at 25 °C. Five mL of Shieh-medium was added on top of Shieh agar plates with
456 confluent lysis and incubated at 7°C for 12-18 h in constant agitation (90 rpm). The lysates
457 were collected, filtered (PES membrane, pore size 0.45 µm, Nalgene®), and stored at +7°C or
458 at –80°C with 20 % glycerol. For phage exposure, phage lysates were diluted with Shieh
459 medium to 5 X 10^5 plaque forming units (PFU) mL^{-1}.
460
461 *Phage exposure experiments and isolation of colonies*
462
463 Two phage-sensitive wild-type *F. columnare* isolates, the high-virulence FCO-F2 isolate
464 (genetic group C) and the medium-virulence FCO-F9 isolate (genetic group G) (This article
465 was submitted to an online preprint archive [36]), were each exposed to three phages in separate
466 experiments with individual phages. Isolate FCO-F2 was exposed to phages FCOV-F2, FCOV-
467 F5 and FCOV-F25, and isolate FCO-F9 to phages FCL-2, FCOV-F13 and FCOV-F45, in

468 accordance with the host range of the phages. Cultures with only bacteria served as no-phage
469 controls. The exposures were carried out in 20 mL of autoclaved fresh water (Lake Jyväsjärvi)
470 in triplicate cultures under constant agitation (120 rpm) at 25ºC for three days at a multiplicity
471 of infection (MOI) at inoculation of 1 (1 X 10^4 CFU and PFU mL^{-1}). The cultures were sampled
472 every 24h for three days, by making a serial tenfold dilution of samples, and spreading on
473 Shieh-agar plates. After up to 4 days of incubation at room temperature, CFUs and colony
474 morphologies were determined from the plate cultures. Two to three colonies from each
475 triplicate culture at each sampling point were picked, and pure-cultured directly on Shieh agar
476 plates three times to get rid of any phage contamination. Colonies were then checked for phage
477 resistance by spot assay on agar plates: bacterial laws on top agar were prepared as above and
478 10 µL of ten-fold diluted original phage lysates (used in initial exposures) were spotted on agar.
479 After 48-h incubation at 25 °C, bacterial plates with no observed plaques or confluent lysis
480 were considered as phage-resistant. Altogether 189 colonies from phage-exposed and no-phage
481 control exposures were isolated from plate cultures. From this collection, 20 phage-exposed
482 and 4 no-phage control isolates were selected for further analysis (Table 2).
483
484 The phage-exposed and no-phage control isolates were named according to the latter part of
485 the wild-type bacterial host, a letter R for phage-exposed and S for no-phage control isolate,
486 plus a running number for the isolated colony. For example, F2R2 is the second selected phage-
487 exposed colony of the *F. columnare* wild-type isolate FCO-F2. Correspondingly, the second
488 *F. columnare* isolate from no-phage control cultures was marked as F2S2. For simplicity, wild-
489 type FCO-F2 and all its subsequent isolates from the phage and control exposures are
490 commonly called F2-isolates in this paper. Correspondingly, wild-type FCO-F9 and its
491 subsequent isolates are called F9-isolates.
492

493 *Antibiotic sensitivity*

494

495 Changes in susceptibility of phage-exposed *F. columnare* isolates towards antibiotics was
496 tested using the Kirby-Bauer disc diffusion method (49) on diluted Mueller-Hinton (50) agar
497 medium supplemented with 5 % w/v fetal calf serum. A 40 µL volume of each isolate
498 suspension (10^9 CFU mL^{-1}) was added to 5 mL phosphate-buffered saline and poured onto the
499 Mueller-Hinton agar plates. After removing excess bacterial suspension by pipetting, the
500 antibiotic discs [oxolinic acid (2 µg), florfenicol (30 µg), sulfamethoxasol/trimethoprim (25
501 µg) and tetracycline (30 µg)] were placed on the plates. The plates were then incubated for 3
502 days at 25°C. After incubation, the inhibition zone around the antibiotic discs was measured.
503 The susceptibility patterns of the selected phage-exposed and no-phage control *F. columnare*
504 isolates to the antibiotics were compared to that of the parent wild-type isolates.

505

506 *Motility/Colony spreading*

507

508 The effect of phage-exposure on bacterial motility was tested by comparing the colony
509 spreading ability of phage-exposed and no-phage control isolates with that of their parent wild-
510 type isolates. After spotting of 5 µL of bacterial suspension (10^9 CFU mL^{-1}) on TYES agar
511 (0.5% agar) plates supplemented with 0.1% baker's yeast and incubation for 3 days at 25ºC,
512 the colony diameter of each isolate was measured. Each isolate was tested in three replicates.

513

514 *Adhesion and biofilm formation*

515

516 Changes in adherence or biofilm formation capacities between wild-type, phage-exposed and
517 no-phage control *F. columnare* isolates were studied in flat-bottomed 96-well microtiter plates

518 (Nunclon Δ Surface, Nunc) (51). *F. columnare* cells grown on TYES agar were suspended in
519 autoclaved fresh water (lake Littoistenjärvi) to a concentration of 10^8 CFU mL^{-1} (OD_{520nm}=0.6).
520 For testing of bacterial adherence, a 100 µL volume of the prepared bacterial suspensions were
521 added in triplicate into wells of replicate microtiter plates and incubated statically for 1 h at
522 25ºC. For testing of biofilm formation, a 100 µL volume of TYES broth was added to wells
523 containing 100 µL of the prepared bacterial suspensions and allowed to incubate for 3 days.
524 Autoclaved fresh water was used as negative control. After incubation, the contents were
525 discarded and the wells were washed three times with sterile 0.5% NaCl to remove non-
526 adherent cells and air dried. The wells were then stained with 0.1% crystal violet solution for
527 45 min and washed three times by submersion in a container of tap water and air dried. The
528 crystal violet was solubilized with 96% ethanol for 15 min before measuring the absorbance (1
529 s) at 595 nm (Victor2, Wallac).
530
531 *Protease activity*
532
533 Changes in protease activity was examined by spotting 1 µL of bacterial TYES broth
534 suspension (10^8 CFU mL^{-1}) of the wild-type isolates and each phage-exposed and no-phage
535 control isolate on TYES agar (1.5% agar) supplemented with (w/v) elastin (0.1%), gelatin (3%)
536 and skim milk (5%) (caseinase production). The proteolytic activity of each isolate was
537 observed by the presence of a clear zone surrounding the colony after incubation, and assessed
538 by measuring the clear zone ratio (diameter of clear zone/diameter of the colony) of three
539 replicate samples. In the absence of a clearing zone outside the colony, the clear zone ratio was
540 defined as 1. The measurements were made after 5 (caseinase and gelatinase) or 10 days
541 (elastinase) of incubation at 25ºC.
542

543 *Virulence*

544

545 Virulence of phage-exposed and no-phage control *F. columnare* isolates was tested on 1.94 g
546 (average weight) rainbow trout fry and compared to the virulence of wild-type isolates. Fifteen
547 fish per treatment, 20 in control treatment with no bacteria, were exposed individually in 500
548 mL of bore hole water (25ºC) to cells of single bacterial isolates by constant immersion (5.0 X
549 10^3 CFU mL^{-1}). Survival of the fish was monitored hourly during 24 h. Morbid fish that did
550 not respond to stimuli were considered dead, removed from the experiment and put down by
551 decapitation. At the end of the experiment, the fish having survived from the infection were
552 put down using 0.008 % Benzocaine. Bacterial cultivations from gills of all the dead fish were
553 made on Shieh agar supplemented with tobramycin (52) to confirm the presence/absence of
554 the bacterium. Cumulative percent mortality and estimated survival time (Kaplan-Meier
555 Survival Analysis), based on observed average survival time of fish after exposure to each
556 isolate, were used as measures of virulence with more virulent isolates having a shorter
557 estimated survival time.

558

559 Fish experiment was conducted according to the Finnish Act of Use of Animals for
560 Experimental purposes, under permission ESAVI/8187/2018 granted for Lotta-Riina Sundberg
561 by the National Animal Experiment Board at the Regional State Administrative Agency for
562 Southern Finland.

563

564 *Whole genome sequencing*

565

566 Genomes of the wild-type FCO-F2 and FCO-F9 *F. columnare* and selected (Table 2) 20 phage-
567 exposed and four no-phage control isolates were sequenced using Illumina HiSeq platform

568 (Institute of Molecular Medicine Finland). The Illumina data reads of FCO-F9 and its phage-
569 exposed and no-phage control isolates were mapped to a reference genome of *F. columnare*
570 isolate B185 (53) using Geneious software version 11.1.5 (Biomatters Ltd.). Genome of the
571 wild-type FCO-F2 isolate was sequenced also using PacBio (BGI, China). PacBio data of FCO-
572 F2 was assembled using > 8kbp reads with Flye (v. 2.7, four iterations) and > 6 kbp with Canu
573 (v. 1.9). These multi-contig assemblies were then combined using Quickmerge (v. 0.3) to
574 produce one 3 221 312 bp contig. This contig was polished with Illumina HiSeq reads using
575 Pilon (v. 1.23), with pre-processing done using Trimmomatic (v. 0.39), bowtie2 (2.3.5.1) and
576 Samtools (v. 1.9). The quality of the polished contig was quantified using Busco (v. 4.0.2),
577 which reported 100% completeness of genome against the bacteria_odb10 reference set. The
578 genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (54,
579 55), and used as reference genome for mapping of F2 phage-exposed and no-phage control
580 isolates.
581
582 *Statistical analyses*
583
584 IBM SPSS Statistics version 24 was used for statistical analysis of the data. A one-way analysis
585 of variance (ANOVA) was used to compare means from phenotypic analyses between
586 experimental groups (phage-exposed isolates and no-phage control isolates) and parent wild-
587 type isolates. If needed, lg10, exponential or square root transformations were made for the
588 data to fulfil the homogeneity of variances assumption. If the homogeneity of variances could
589 not be met by transformations, the data were analysed using non-parametric Kruskal-Wallis
590 and Mann-Whitney tests. In case of elastinase and casienase activity, and biofilm formation,
591 the isolates with no activity/biofilm forming capacity were excluded from the ANOVA LSD

592 multiple comparison analyses. Kaplan-Meier Survival Analysis was used for analysis of
593 virulence data.
594
595 *Data availability*
596
597 The whole genome sequences of all the isolates were submitted to GenBank under accession
598 numbers presented in Table 3.
599
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601
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605
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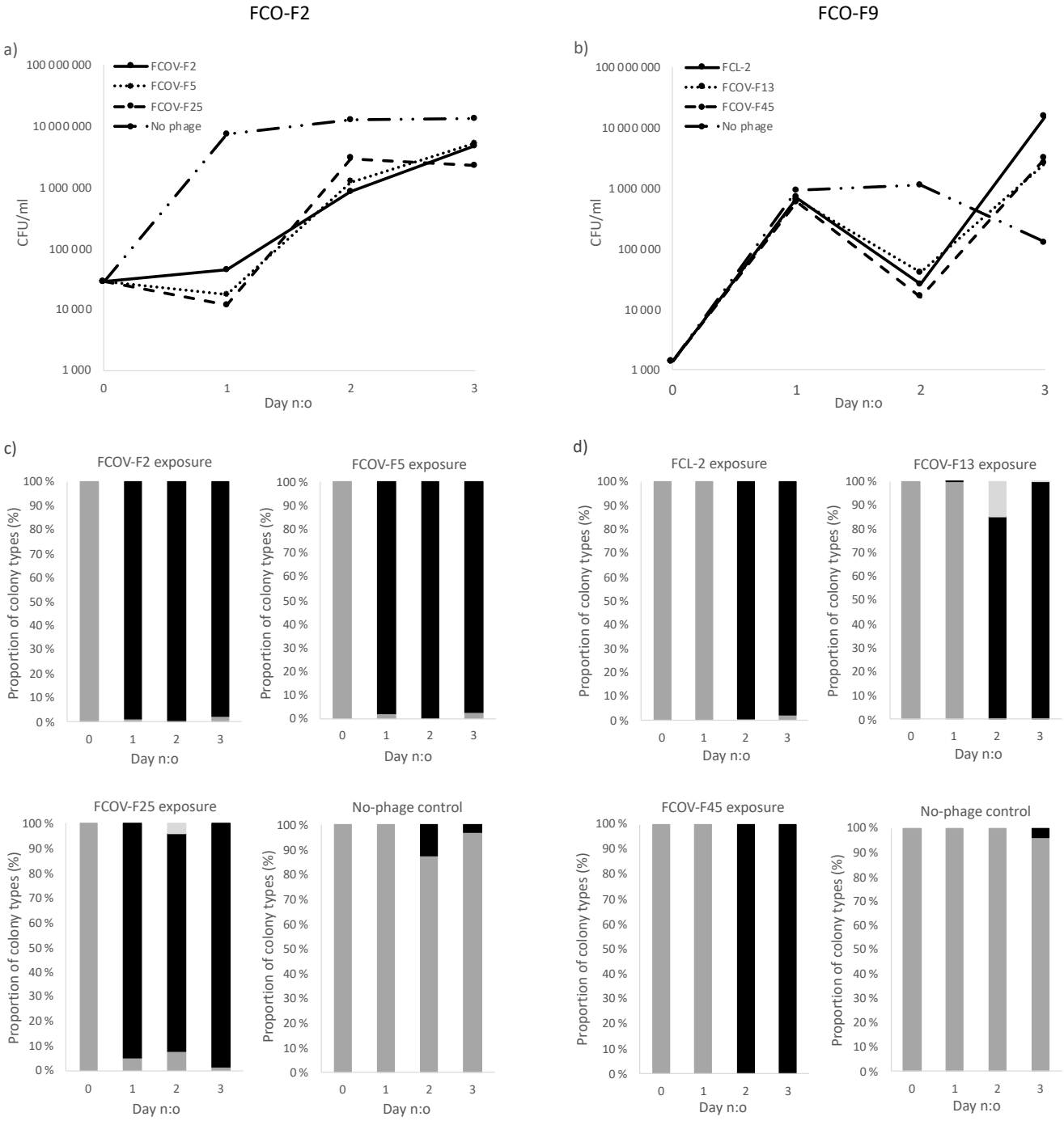
764

765 **Figures**



766

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

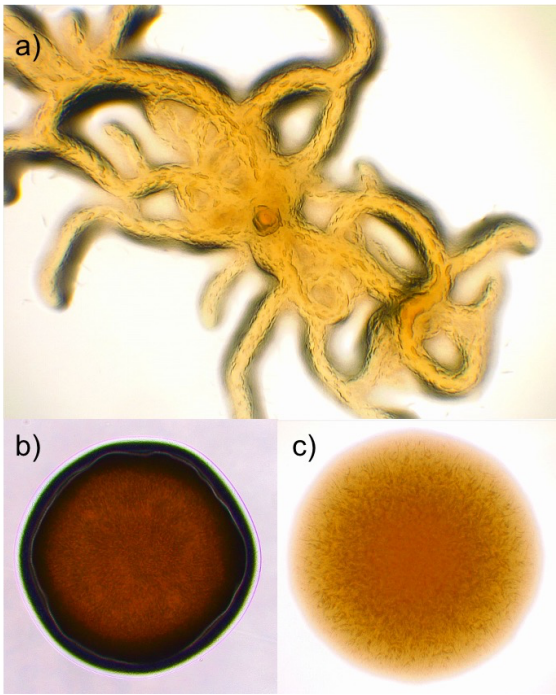


Figure 2. Different colony morphologies formed by *Flavobacterium columnare* on Shieh-agar plates after phage exposure: a) rhizoid, b) rough and c) soft.

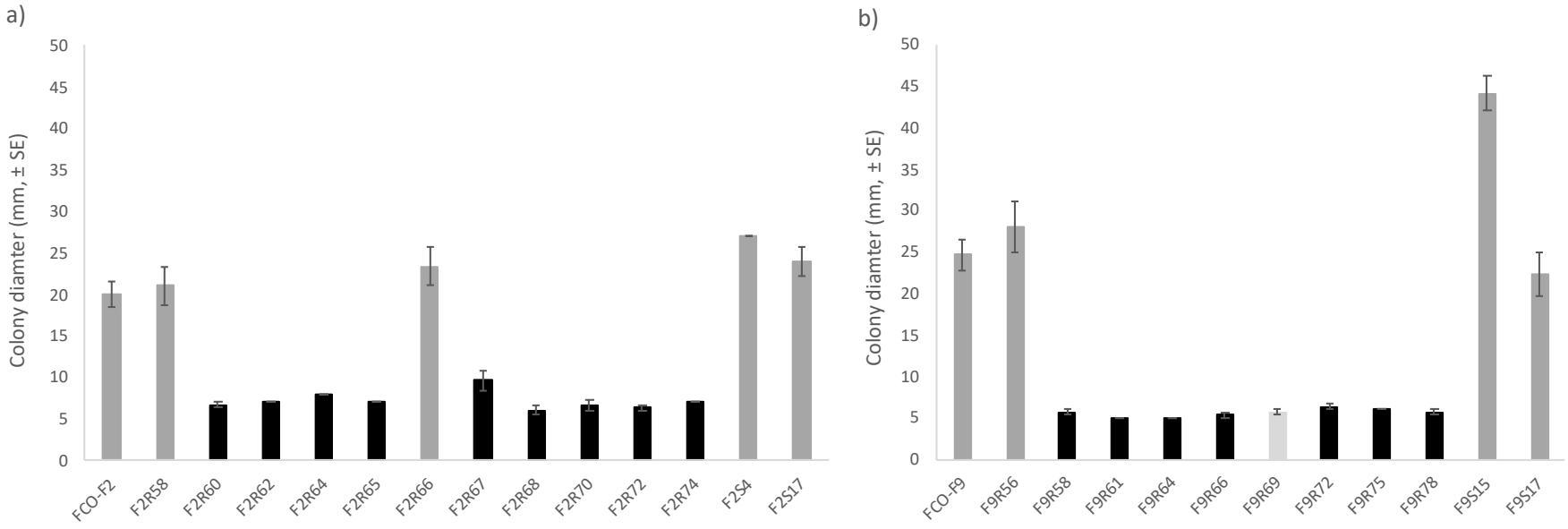


Figure 3. Motility of *Flavobacterium columnare* wild-type a) FCO-F2 and b) FCO-F9 isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates expressed as colony diameter (mm, ±SE) on TYES agar. All the phage-sensitive rhizoid colonies forming isolates (dark grey 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).

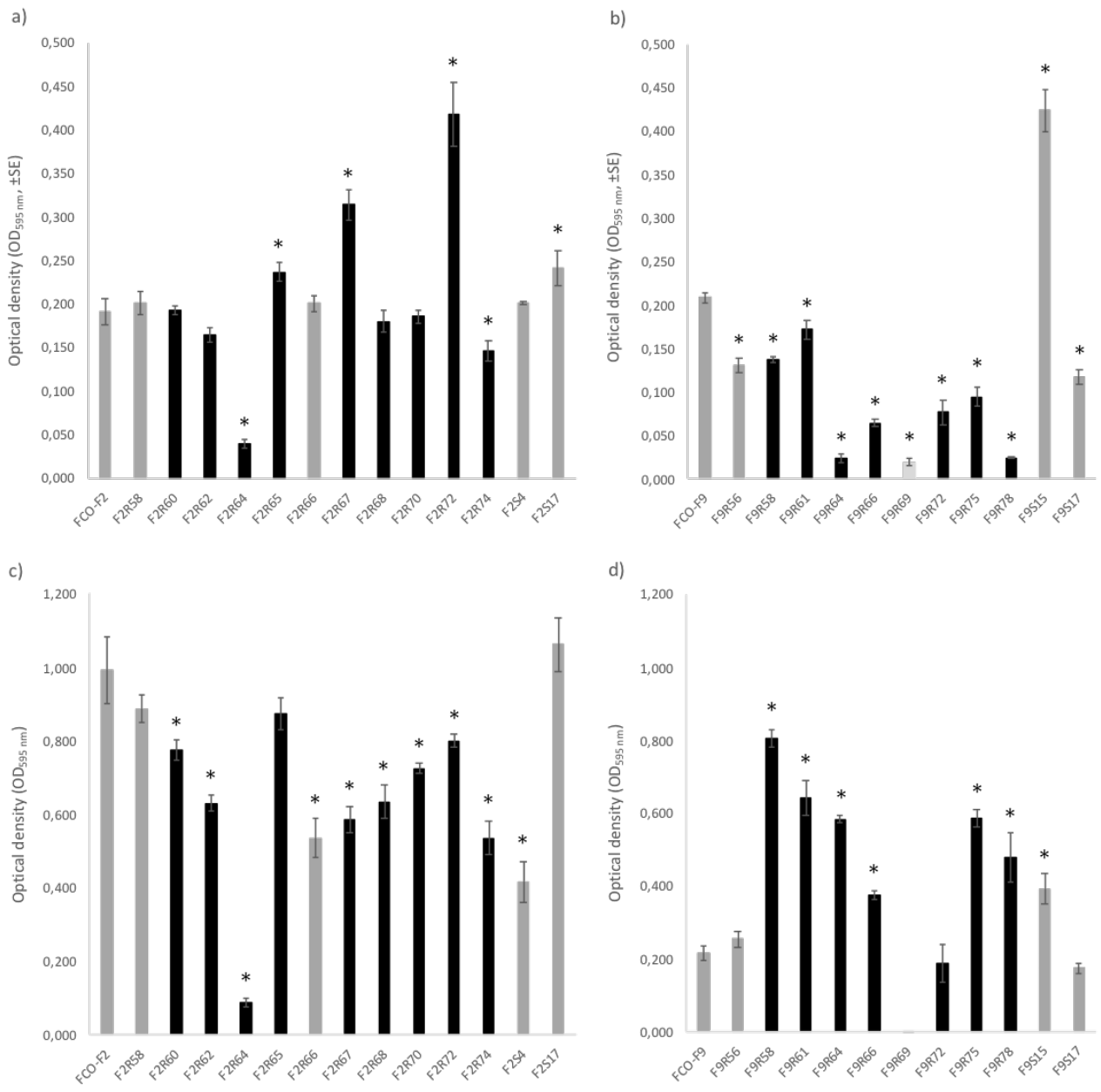


Figure 4. Adherence (a and b) and biofilm forming (c and d) capacity of *Flavobacterium columnare* wild-type FCO-F2 (a and c) and FCO-F9 (b and d) isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates on a polystyrene surface measured as optical density ($OD_{595\ nm}$, ±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.

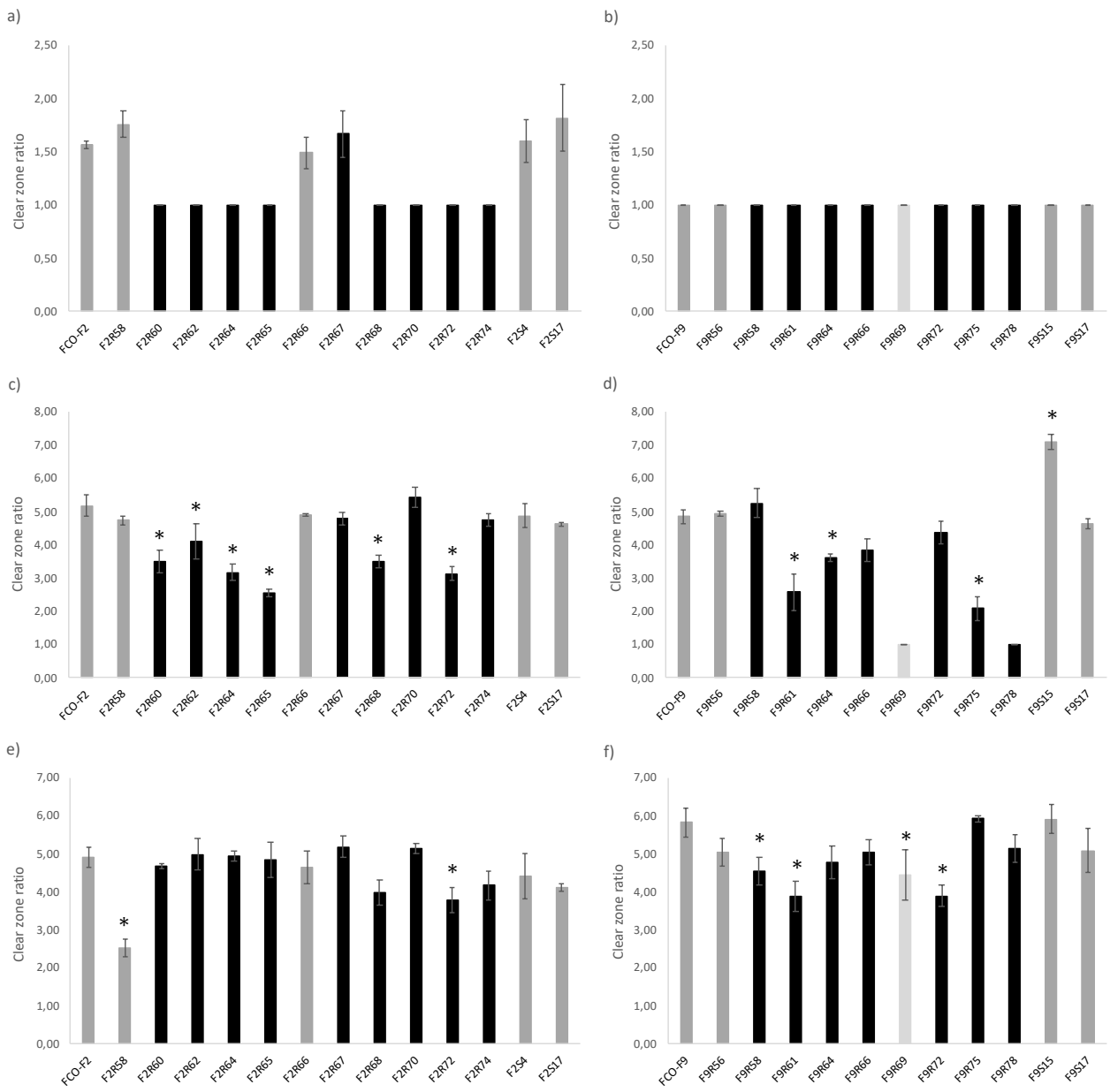


Figure 5. Protease (elastinase: a and b, gelatinase: c and d, and caseinase e and f) activity of the *Flavobacterium columnare* FCO-F2 (a, c and e) and FCO-F9 (b, d and f) isolates, and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates. The activity was measured as the 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.

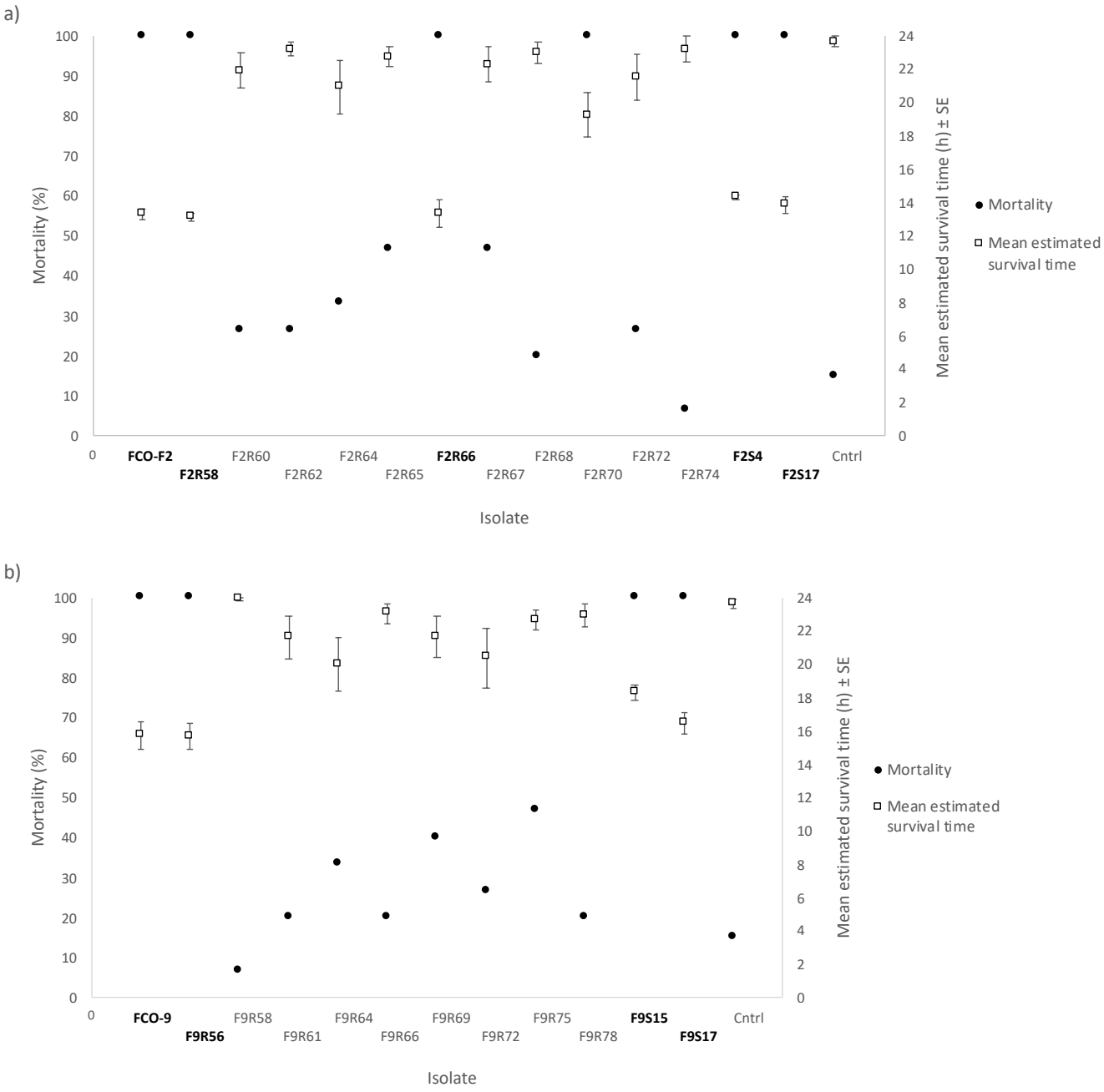


Figure 6. Mortality percent and estimated survival time (± SE) of rainbow trout (*Oncorhynchus mykiss*) during 24-h experimental infection with wild-type *Flavobacterium columnare* FCO-F2 (a) and FCO-F9 (b), and their phage-exposed (F2R- and F9R-) and no-phage control (F2S- and F9S-) isolates. Phage-sensitive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

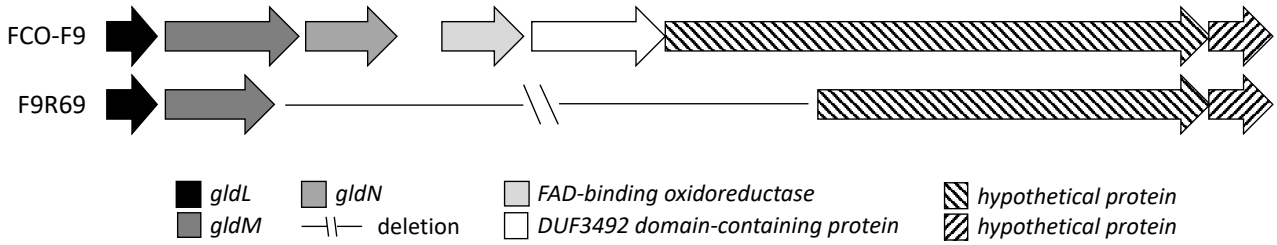


Figure 7. Deletion of genomic region covering 4 701 bp in FCOV-F13 exposed, soft colonies forming, phage-resistant *Flavobacterium columnare* isolate F9R69.

923 **Tables**
924
925 **Table 1.** *Flavobacterium columnare* isolates and phages used in this study. Bacteria and
926 phages were isolated from Finnish fish farms. *F. columnare* isolates have previously been
927 categorized into genetic groups by restriction fragment length polymorphism analysis of
928 internal transcribed spacer region between 16S and 23S rRNA genes (This article was
929 submitted to an online preprint archive [36]).
930

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

941

Table 2. 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	+	+	+

Table 3. 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

1010 **Table 4.** Data on genomes of wild-type *Flavobacterium columnare* strains FCO-F2 and
1011 FCO-F9.
1012

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

Table 5. 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 6. 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