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1 **Effects of resource availability on evolution of virulence and competition in an**
2 **environmentally transmitted pathogen**
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34 # Abstract
35

36 Understanding ecological and epidemiological factors driving pathogen evolution in
37 contemporary time scales is a major challenge in modern health management.
38 Pathogens that replicate outside the hosts are subject to selection imposed by ambient
39 environmental conditions. Increased nutrient levels could increase pathogen virulence
40 by pre-adapting for efficient use of resources upon contact of a nutrient rich host or by
41 favouring transmission of fast-growing virulent strains. We measured changes in
42 virulence and competition in *Flavobacterium columnare,* a bacterial pathogen of
43 freshwater fish, under high and low nutrient levels. To test competition between
44 strains in genotype mixtures, we developed a quantitative real-time PCR assay. We
45 found that the virulent strain maintained its virulence and outcompeted less virulent
46 strains independent of the nutrient level and resource renewal rate, but a less virulent
47 strain further lost virulence in flow-through chemostats under low nutrient level and
48 in long serial culture under high nutrient level. Our results suggest that increased
49 outside-host nutrients might maintain virulence in less virulent strains and increase
50 their contribution in epidemics in aquaculture. The results highlight a further need to
51 study the role of resources in the outside-host environment in maintaining strain
52 diversity and driving evolution of virulence among environmentally growing
53 pathogens.
54

55 Key words: bacterium, *Flavobacterium columnare*, fish disease, interference
56 competition, qPCR, resource competition
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66 ## Background
67
68 Evolution can have a significant impact on properties and responses of organisms
69 within ecological time scale over a few tens or hundreds of generations (Geerts, et al.
70 2015, Stuart, et al. 2014, Yoshida, et al. 2003). These effects can be especially
71 pronounced in organisms with short generation times, such as microbes (Hiltunen, et
72 al. 2014). Evolutionary changes resulting in more serious disease and epidemics are
73 presenting major challenges for health management (Griette, et al. 2015), and
74 emphasize the importance of understanding the ecological and epidemiological
75 factors underlying the evolutionary changes for disease control (Bull and Lauring
76 2014, Cressler, et al. 2016, Kennedy, et al. 2016, Lively, et al. 2014).
77
78 Hosts and the nutrition they offer are among the fundamental selective environments
79 encountered by pathogens. However, in pathogens capable of environmental growth
80 and replication, the outside-host environment might be equally important by
81 favouring phenotypic properties that increase fitness in that particular environment
82 (Brown, et al. 2012) and selecting for genotypes conferring these properties (New, et
83 al. 2014). Thus outside-host conditions can be important for ecological and
84 evolutionary trajectories for environmentally replicating opportunistic pathogens.
85
86 Microbial competitive ability in a particular resource environment is mainly governed
87 by resource utilization traits such as growth rate (Litchman, et al. 2015). For
88 environmentally growing bacteria, fast growth is essential in fighting against
89 competing microbes, but will also increase the probability of being the first to infect a
90 host upon contact. High resource availability may therefore increase the share of fast
91 growing strains in host infections.
92
93 Resource environment may also direct selection on resource utilization traits
94 (Litchman, et al. 2015) and induce diversification in resource use (Cooper and Lenski
95 2000, Jasmin and Kassen 2007, Szappanos, et al. 2016). Resource utilization traits can
96 be traded off against each other, such that adaptation to one resource environment
97 may decrease the competitive ability of a microbe on alternative resources (Cooper
98 and Lenski 2000, Litchman, et al. 2015). On the other hand, growth in matching
99 outside-host resources might shorten the lag-phase in growth and speed up the ability

100 to exploit host resources (Brown, et al. 2012, Ketola, et al. 2016, Velicer and Lenski
101 1999). There is evidence that virulence genes are affected by nutrient environment,
102 such that growth in rich resources in the outside host environment could prime
103 environmentally growing opportunistic pathogens for higher virulence towards the
104 host (Brown, et al. 2012, Ketola, et al. 2016, Rohmer, et al. 2011).
105
106 Intensive farming is suggested to favour increase in pathogen virulence for example
107 via increased contact rates, fast turnover of hosts and strain competition (Kennedy, et
108 al. 2016, Mennerat, et al. 2010, Pulkkinen, et al. 2010). However, in addition to
109 epidemiological factors, intensive farming environments contrast the natural
110 environment with regard to ambient conditions such as resource availability for
111 environmentally growing microbes. Nutrient enrichment is increasingly being
112 connected with increase in infectious and parasitic diseases (Aalto, et al. 2015).
113 Recent work has also shown the potential of higher resource availability in the
114 outside-host environment to induce increased virulence on environmentally growing
115 opportunistic pathogens (Kinnula, et al. 2017b, Penttinen, et al. 2016, Wedekind, et
116 al. 2010).
117
118 Increased virulence at fish farms over the course of last 40 years has been suggested
119 for *Flavobacterium columnare,* an environmentally growing bacterial pathogen of
120 freshwater fish (Kunttu, et al. 2012, Suomalainen, et al. 2006a), potentially due to
121 selection for certain genotypes of the bacterium (Pulkkinen, et al. 2010, Sundberg, et
122 al. 2016). Apart from diseased fish, *F. columnare* can be frequently isolated from lake
123 water and biofilms (Kunttu, et al. 2012), and the bacterium can efficiently transmit to
124 a new host also from dead fish (Kunttu, et al. 2009b). Higher ambient nutrient
125 concentration leads to increased virulence in fish challenge experiments (Kinnula, et
126 al. 2017b, Penttinen, et al. 2016). In aquaculture, high fish densities increase water
127 nutrient levels due to fish excretion, faeces and uneaten fish feed (Lalonde, et al.
128 2015). A positive association between growth rate and virulence (Pulkkinen, et al.
129 2010) could indicate potential for selection towards higher virulence under high
130 nutrient availability for this environmentally growing pathogen.
131
132 Here we examined the hypothesis that high nutrient levels in the outside-host
133 environment in fish farms select for more virulent strains of *F. columnare* by 1)

134 increasing the overall virulence of the strains towards fish hosts and/or 2) favouring
135 virulent strains over less virulent strains in competition. To study the first question,
136 we first cultured a virulent and a less virulent strain under low and high nutrient level
137 in monocultures. After evolving under certain nutrient level, we tested if the overall
138 virulence in the population had changed in comparison to ancestral strains (fish
139 challenge tests *in vivo*). In order to study the second question on the effect of nutrient
140 level on competition between the pair of a virulent and a less virulent strain, we
141 initiated co-cultures with a 1:1 ratio of each strain and followed genotype frequencies
142 using real-time quantitative PCR (qPCR) assay developed for this purpose. We also
143 tested growth inhibition between the strains, because interference (direct) competition
144 is common for *F. columnare* growing on surfaces (Ashrafi, et al. 2017, Sundberg, et
145 al. 2016).
146
147 We performed two separate experiments, each with the same virulent but a different
148 less virulent bacterial strain. The two experiments differed in the rate of resource
149 renewal. In the first experiment, bacteria were inoculated into batch cultures and only
150 the small volume of culture removed upon sampling was replaced with fresh medium
151 (low rate resource renewal). In the second experiment, high rate resource renewal was
152 executed at high nutrient level with daily serial transfer of a small aliquot of bacterial
153 culture to fresh growth medium in test tubes and at low nutrient level by constant
154 flow-through of the medium in chemostats (Velicer and Lenski 1999). In the high rate
155 renewal experiment, the co-cultures initiated from ancestral strains were terminated
156 after three weeks, when the virulent strain had outcompeted the less virulent strain.
157 While the monocultures were maintained as they were, another set of co-cultures was
158 initiated using bacteria evolved at the respective nutrient level in monocultures in
159 order to study if potential adaptation to a nutrient level changes the competition
160 outcome. For the high rate resource renewal experiment we also tested if biofilm
161 formation of the strains explain competition outcome.
162
163 We expected that cultivation in high nutrient level would pre-adapt the bacteria for
164 fast exploitation of nutrient rich host and therefore increase their virulence in fish
165 challenge tests. Concerning competition, we expected that the virulent strain would
166 grow faster (Pulkkinen, et al. 2010) and outcompete a less virulent strain under high
167 nutrient level. However, under low nutrient level, the slow-growing less virulent

strain was expected to win because the replication of the virulent strain should be restricted by the high nutrient requirements of a high growth rate (Hibbing, et al. 2010).

Methods

Bacterial strains and culture conditions

The virulent strain B402 was isolated from a diseased fish at a fish farm. It was compared pairwise in two separate experiments with a less virulent strain (B407 or B398) isolated from river water (Kunttu, et al. 2012). The virulence of these strains had been tested previously in fish challenge experiments (Kunttu, et al. 2012). Strains were grouped in genotypes based on ARISA (automated ribosomal intergenic spacer analysis) (Kunttu, et al. 2012), and more recently on MLSA (multilocus sequence analysis) (Ashrafi, et al. 2015).

All strains were preserved as stock cultures containing 10% foetal calf serum and 10% glycerol at -80°C. The stocks were revived by incubation in 5 mL of modified Shieh medium (from now on Shieh medium) (Song, et al. 1988) at room temperature and constant shaking (120 RPM) for 24 hours and subsequent renewal in 1:10 for another 24 hours.

Bacterial cultures were grown either under low or high nutrient level. For each nutrient level, the Shieh growth medium was adjusted to the desired concentration with sterile water. Water, rather than nutrient-free saline buffer was used, because of low tolerance of *F. columnare* to salt water (Suomalainen, et al. 2006a). Bacterial densities used in inoculation were determined by measuring the optical density of the revived culture at 595 nm and comparing to a previously determined relationship between optical density and colony forming unit (CFU mL^{-1}) counts. During the experiment, the bacterial growth in the cultures as CFU mL^{-1} was quantified by serial dilution and plate cultivation on Shieh-agar.

Batch culture experiment (low rate resource renewal)

200 In batch cultures, one-tenth of culture volume was replaced with fresh medium upon
201 sampling. Bacteria (a virulent strain B402 and a less virulent strain B407) were
202 grown in 5% (low nutrient level) and 50% (high nutrient level) Shieh medium. The
203 total culture volume was 30 mL in 50 mL plastic test tubes, three replicates per strain
204 or co-culture. The estimated starting number for bacteria was 1.0×10^4 CFU mL^{-1} for
205 the monocultures and 0.5×10^4 CFU mL^{-1} for each of the two strains (1:1 ratio) for the
206 co-cultures. The batch cultures were kept in a shaker incubator under constant
207 agitation (120 RPM) at 25 °C.
208
209 Samples were taken from all cultures on days 0-5, 7, 9, 11, 16, 21 and 35 (see
210 supplementary Table S1). Each sampling day, 3.1 mL samples were taken from each
211 tube and replaced with fresh medium. One hundred µL was used for plate cultivation
212 for both mono- and co-cultures. For co-cultures, in order to determine the proportions
213 of two bacterial strains in the sample, 3 mL was used for DNA extraction for qPCR
214 (see below). On day 21, samples collected from the bacterial populations evolved in
215 monocultures were tested for virulence in a fish challenge experiment *in vivo* and
216 compared to ancestral isolates (see below).

217 **High rate resource renewal experiment**
218

219 High rate resource renewal was executed with continuous supply of low nutrient level
220 (2%) Shieh medium in chemostats (a flow-through microcosm) or with daily serial
221 transfer in high nutrient level (20%) Shieh medium in test tubes (Velicer and Lenski
222 1999). The concentration of Shieh medium at the low nutrient level could not be kept
223 at 5% used in the batch experiment because it blocked the chemostat tubes, but was
224 decreased to 2%. Consequently, the concentration in the high nutrient level was
225 decreased from 50% to 20%, in order to keep the relative difference between low and
226 high nutrient level the same, i.e. 10 x differences. The strains used were B402
227 (virulent) and B398 (less virulent). The chemostat volume was approximately 455
228 mL. The chemostats were assembled aseptically and magnetic stirrer bars were placed
229 at the bottom of the chemostats. A constant flow of fresh 2% Shieh medium was
230 supplied from sterile 5 L flasks to the bottom of the chemostat with a Gilson
231 peristaltic pump at dilution rate 0.21-0.24 day^{-1}. During the experiment, the medium
232 bottle was replaced at ca. 20 day intervals. The overflow from the top of the

233 chemostat was collected via tubing to a separate waste bottle and discarded. For high
234 nutrient level, the total culture volume was 30 mL in 50 mL plastic test tubes. In order
235 to maintain the bacterial population in exponential growth phase, a 50 μL sample was
236 transferred to a new tube containing 30 mL of fresh medium each day. The culture
237 tubes were kept under constant agitation (120 RPM), in the same room as the
238 chemostats, with temperature adjusted at 22.6 ± 0.1°C. Each tube culture was
239 replicated three times and each chemostat twice.
240
241 The monocultures were maintained continuously for 53 days (Table S1). Samples
242 collected from the evolved populations on days 24 and 53 were stored at -80°C as
243 described above and later tested for virulence in a fish challenge experiment *in vivo* in
244 comparison to ancestral isolates (see below). Co-cultures were started with ancestral
245 strains simultaneously with the monocultures, however, when the virulent strains
246 became dominant during the first two weeks at both nutrient levels in all replicates
247 and no further change was seen within the next week (see Fig. 2B), co-cultures were
248 discarded at day 20. A second set of co-cultures were initiated with samples taken
249 from monocultures on day 25 and, for one chemostat - due to a fail in first inoculation
250 - on day 31 (see below, Table S1). The idea of the second set of co-cultures was to
251 study if the competitive outcome would change after the strains had already been
252 adapting to a certain nutrient level. The second set of co-cultures was maintained for
253 28 days.
254
255 The estimated starting number for bacteria for the batch monocultures was $1.0x10^4$
256 CFU mL^{-1} and $0.5x10^4$ CFU mL^{-1} for each of the two strains (1:1 ratio) in co-
257 cultures. Chemostat monocultures were inoculated with $1.0x10^6$ CFU mL^{-1} and the
258 first co-cultures with $0.5x10^6$ CFU mL^{-1} of each of the two strains. The second co-
259 cultures were inoculated with bacteria taken from the monocultures and diluted to
260 desired bacterial concentration using the data from last preceding CFU-
261 measurements. The concentration of bacteria in one of the monocultures used for
262 inoculation was so low that one co-culture had to be started with lower concentration
263 than aimed, with a total concentration of $5.0x10^4$ CFU mL^{-1} ($2.5x10^4$ CFU mL^{-1} of
264 each strain). Another co-culture started at the same time crashed and was re-started 6
265 days later with a total concentration of $1.0x10^6$ CFU mL^{-1} ($0.5x10^6$ CFU mL^{-1} of each

266 of the two strains). In comparison of growth and proportion of strains in competition
267 in co-cultures, the days since inoculation were used (see Table S1).
268
269 For tube cultures, the plate counting was done from the culture remaining after the
270 daily transfer of 50 μL to fresh medium and for chemostats from samples taken with a
271 sterile needle and syringe through two ports near the top and bottom of the chemostat
272 (total sample volume approximately 40 mL) mixed in equal proportions. For co-
273 cultures the remaining culture in the tube and chemostat sample were used for DNA-
274 extraction and subsequent qPCR (see below).
275

276 Biofilm formation in the high rate resource renewal experiment

277
278 In order to evaluate if differences in relative amount of biofilm formation between the
279 virulent and less virulent strain in the high rate resource renewal experiment affected
280 their growth and competition, we quantified the relative amount of biofilm formed
281 during 48 hour incubation in low or high nutrient level. Bacterial cultures (strains
282 B402 and B398) were inoculated into 200 μL of 2% or 20 % Shieh medium ($1.0x10^6$
283 and $1.0x10^4$ CFU mL^{-1}, respectively) in eight replicate wells per strain x nutrient level
284 on 96 well spectrophotometer plates. After 48 h, the wells were emptied, rinsed with
285 distilled water, and the biofilm bound to the walls of the wells were dyed with 200 μL
286 of 1% crystal violet solution. After 10 min, the wells were emptied with swift shaking
287 of the overturned plate and rinsed by filling with distilled water. Rinsing and
288 emptying was repeated three times and after the last rinse, the wells were visually
289 confirmed to be dry. 96% ethanol was added to dissolve the dye. After 24 h, the
290 optical density was measured with a spectrophotometer with 570 nm wavelength.

291 Assessment of genotype frequencies in co-cultures

292
293 To evaluate the frequency of virulent and less virulent strain in co-culture we
294 developed a quantitative PCR (qPCR) assay including primer design and tests of
295 primer specificity and efficiency. We used *F. columnare trpB* (tryptophan synthase
296 subunit B) sequences (Ashrafi, et al. 2015) to design specific primers that amplify
297 genotype C DNA only, and universal primers that amplify all five genotypes found in
298 Finland (genotypes A, C, E, G and H). The proportions of two bacterial strains in co-

299 culture were then determined using ΔCq (Briand, et al. 2008). In short; standard
300 curves were prepared using samples containing known proportion of the virulent
301 strain (B402, genotype C) DNA mixed together with the less virulent strain (B407,
302 genotype G, or B398, genotype A) DNA and by plotting the proportion of the virulent
303 strain against the ΔCq value of the sample ($\Delta Cq = Cq_{specific\ primers} - Cq_{universal\ primers}$).
304 The PCR-reactions with specific and universal primers were always run within the
305 plate and the ΔCq values were calculated using the mean of three or sometimes two
306 technical replicates (outliers were removed from the data, sometimes leaving only two
307 of the three replicates for analysis). The unknown samples were run similarly,
308 together with a positive control containing DNA of the strains in 1:1 proportion. The
309 positive control was used to normalize the ΔCq values of the samples before
310 determining the proportion of the virulent strain in a sample using the constructed
311 standard curve. For further details, including DNA extraction and qPCR assay
312 validation, see supplementary methods, tables S2-S3 and figures S1-S3.

313 **Interference competition**
314

315 To evaluate the potential for interference competition via excreted products in
316 explaining the outcome of competition in co-cultures, we studied the growth
317 inhibition between the pairs of strains used in the experiments with the double layer
318 method (Sundberg, et al. 2016). Shortly, an aliquot of bacterial culture was
319 centrifuged (17 000 g, 3 min) and five μL of the supernatant was pipetted on top of
320 Shieh agar plate, where 300 μL of the other bacterium strain had been poured after
321 mixing with soft Shieh agar (0.7%). Three separate replicates were prepared for each
322 strain. Plates were checked after 48 h to detect if the supernatant had inhibited the
323 growth of the underlying strain.
324

325 **Fish challenge experiments**
326

327 Prior challenge experiments, ancestral strains were revived from frozen stock cultures
328 and all bacterial samples from different nutrient levels were subcultured in 100%
329 Shieh as described above (basic culture conditions). Thus the outcome of the
330 challenge tests were likely not affected by the nutrient levels added to the fish
331 containers.

332

333 Challenge experiments were performed with rainbow trout fingerlings obtained from
334 a fish farm using ground water, ensuring that the fish had no previous exposure to *F.*
335 *columnare*. The fish were maintained in the laboratory in aerated well water in glass
336 aquaria at 12:12 L:D cycle at 17°C and fed with commercial pellets. The water
337 temperature was increased gradually to 25°C (1-1.5 °C per day) during ten days
338 before the bacterial challenge. The fish were transferred individually into transparent
339 plastic containers with 0.5 L of aerated well water at 25°C. The containers were
340 randomly assigned to different treatments (10 replicates per treatment) and the
341 cultures, adjusted to 1.5E+03 CFU ml^{-1} final concentration, were added to the
342 containers in 0.1mL of 100% Shieh according to continuous challenge method
343 (Kinnula, et al. 2015). The fish were monitored at one hour interval for signs of
344 bacterial infection and morbidity. Due to fast development of disease symptoms, the
345 diseased fish were mostly not responding to external stimuli and were killed by
346 cutting the spinal cord with scissors leading to instant death. A bacterial sample was
347 taken from the gills with a sterile loop and spread on agar plates containing modified
348 Shieh medium and tobramycin (Decostere, et al. 1997). Fish weight in mg was taken
349 down. The fish surviving until the end of experiments were euthanized with an
350 overdose of MS-222, sampled for bacteria on gills and weighed.

351

352 Columnaris disease is a threat especially to young salmonids, with a rapid progress of
353 the disease at freshwater fish farms. Challenge experiments were performed with
354 rainbow trout fingerlings (*Oncorhynchus mykiss*) (age 0+) with a mean weight
355 2.10±0.69 g when testing bacteria from the batch experiment and 0.39±0.12 g when
356 testing bacteria from the high rate resource renewal experiment. For this size range,
357 previous studies have confirmed a qualitatively similar progress of infections
358 (Kinnula, et al. 2015, Laanto, et al. 2014, Sundberg, et al. 2014). The experiments
359 were ended after 45 h (batch experiment) and 49 h (high rate resource renewal
360 experiment). The fish challenge experiments were conducted according to the Finnish
361 Act on the Use of Animals for Experimental Purposes under the license number
362 ESAVI/10184/04.10.07/2014 granted to Jouni Taskinen by the National Animal
363 Experiment Board of the Regional State Administrative Agency of Southern Finland.

364

365 **Statistical analyses**
366
367 **Competition and growth**
368
369 The data was analysed separately for the batch experiment and the high rate resource
370 renewal experiment. For the high rate resource renewal experiment the data was
371 divided in two parts according to the duration of the two sets of co-cultures (days 0-24
372 and 25-53). The data was analysed in R version x 64 3.4.3 with the package
373 MCMCglmm (Hadfield 2010) with Bayesian mixed models using Markov chain
374 Monte Carlo estimation. To study the outcome of competition in co-cultures using
375 qPCR-data, ratio of the virulent strain in competition was analysed by using nutrient
376 level (low vs. high) as a fixed factor and day since inoculum as a continuous factor.
377 The non-independence of multiple observations from the same replicate was
378 accounted for by including replicate identity as a random factor. The bacterial growth
379 (CFU ml^{-1}) was compared between monocultures and co-cultures using nutrient level
380 (low vs. high) and strain or strain combination (virulent, less virulent, co-culture) as
381 fixed factors. Day since inoculum was included as a continuous covariate and
382 replicate identity as a random factor. The models for studying competition from qPCR
383 data were fitted with Gaussian distribution and the effect of nutrient level on growth
384 were fitted using Poisson distribution to account for overdispersion of the data. As
385 model selection with DIC criteria might not be reliable for non-Gaussian data, the
386 models were fitted including all main effects and 2-way interactions. For part 1 (days
387 0-24) in high rate resource renewal experiment also 3-way interactions were included.
388 Inverse-Wishart priors with a low degree of information were used for both fixed and
389 random effects (Hadfield 2010). Diagnostic tools from the Coda package (Plummer,
390 et al. 2006) were used for determining the number of the iterations (600 000), length
391 of thinning (130) and burn-in period (100 000) of the models. Gelman-Rubin test and
392 visual inspection of traces on three chains were used for checking model convergence.
393 The potential scale reduction factor was close to 1 among chains in all analyses.
394 Differences between factors are considered significant if the 95% credible intervals
395 for the posterior means do not overlap with zero.
396
397 In order to evaluate if the outcome of competition is affected by differences in growth
398 between the strains in different nutrient levels in the beginning of the experiments

399 (days 0-1), initial population growth was calculated for each monoculture as
400 $\ln(N_{day1}/N_{day0})$/1 day (Lenski, et al. 1991). N_{day0} was the initial inoculation density and
401 N_{day1} the population density on day 1 determined by plate counting. These values
402 were compared between the virulent and less virulent strain within each nutrient level
403 separately for the batch experiment and the high rate resource renewal experiment
404 with ANOVA.
405

406 **Biofilm formation**
407

408 For evaluation of the effect of biofilm formation on the outcome of growth and
409 competition in the high rate resource renewal experiment, the optical density values
410 obtained from the separate biofilm experiments between the virulent and less virulent
411 strain under low and high nutrient level were compared with ANOVA after log-
412 transformation.

413 **Fish challenge tests**

414
415 For analysing data from *in vivo* fish challenge experiments, generalized linear models
416 with binomial distribution were used to study the effect of bacterial strain identity and
417 treatment (ancestral isolate, evolved under low or high nutrient level) on the
418 morbidity of fish. The analyses were performed separately for the batch experiment
419 and for the high rate resource renewal experiment. Strain and treatment were included
420 as fixed factors. Fish weight was included as a continuous covariate to control for the
421 effect of surface area and respiration rate on infection risk of a fish. Model selection
422 was based on Akaike information criteria, performed with stepAIC function from
423 package MASS in R (Venables and Ripley 2002), starting from the full model
424 including all 3-way interactions. In the challenge experiment for bacteria evolved in
425 the high rate resource renewal experiment, samples collected from the cultures on day
426 24 and day 52 were considered independent of each other and included as fixed
427 treatment factors (ancestor, high nutrient day 24, high nutrient day 52, low nutrient
428 day 24, low nutrient day 52).

Results

Fish challenge experiments

Batch culture experiment

Culturing bacteria in batches with low rate resource renewal at low or high nutrient levels did not change the virulence of the bacteria towards rainbow trout fingerlings in the challenge experiment as compared to the ancestral isolates. The best model explaining the outcome of the challenge experiment according to the AIC criteria included only the effect of strain (Table 1). The virulent strain B402 caused higher morbidity in rainbow trout than the less virulent strain B407 in all treatments (Fig. 1A, Supplementary Fig. S4A).

High rate resource renewal experiment

Populations evolved under high rate resource renewal in low or high nutrient levels for different lengths of time (24 days or 52 days) were compared to ancestral isolates in their ability to induce morbidity in fish. The best model according to AIC criteria included interaction between the strain (virulent, less virulent) and the treatment (ancestral, low nutrient at day 24 or 52, or high nutrient at day 24 or 52). The morbidity caused by the virulent strain B402 was higher than that caused by the less virulent strain B398. The nutrient level did not affect the morbidity caused by the virulent strain B402. However, for the strain B398, which was originally less virulent, morbidity in fish decreased after culturing as compared to that caused by ancestral strain. This was especially evident after culturing under low nutrient level but also after culturing at the high nutrient level for the longer time period (Table 2, Fig. 1B, Supplementary Fig. S4B).

Competition and growth

460 **Batch experiment**
461
462 The proportion of the virulent strain determined with the qPCR assay did not differ
463 between high and low nutrient level (MCMCglmm model: the 95% credible intervals
464 for the posterior means for the low nutrient level and interaction between low
465 nutrient: day overlapped zero; Table S4, Fig. 2A). The proportion of the virulent
466 strain increased during the experiment as indicated by the positive deviance of day
467 from zero (posterior mean for day 0.003, 95% CI:0.001-0.005).
468
469 The bacterial concentrations of the virulent strain in monoculture, the less virulent
470 strain in monoculture and the competition treatment did not differ from each other,
471 they did not change during the experiment and the nutrient level did not affect the
472 concentrations (Table S4, Fig. 3A-B). This is shown by the posterior distribution
473 credible intervals which overlapped with zero for all variables considered in the
474 MCMCglmm model.
475
476 The initial population growth during the first 24 h showed different patterns among
477 the strains in the monocultures in the low and high nutrient levels (ANOVA: strain
478 $F_{1,8} = 59.139$, $p < 0.001$, nutrient $F_{1,8} = 89.048$, $p < 0.001$, nutrient x strain effect $F_{1,8} =$
479 28.010, $p < 0.001$). The initial population growth was higher in the monocultures of
480 virulent strain than for the less virulent strain in the high nutrient level, but there was
481 no difference between the strains in the low nutrient level (pairwise comparisons,
482 supplementary Table S7). Both strains had higher initial growth when grown in high
483 nutrient level than under low nutrient level (Table S7).
484

485 **High rate resource renewal experiment**
486
487 When the co-cultures were started from ancestral strains, the proportion of the
488 virulent strain did not differ between high and low nutrient level (MCMCglmm
489 model: 95% CI for the posterior means overlapped zero for the low nutrient level and
490 the interaction between low nutrient level: day; Table S5, Fig. 2B). The proportion of
491 the virulent strain increased from the initial 50% to approximately 100% during the
492 experiment as indicated by the positive deviance of day from zero (MCMCglmm
493 model: 0.020, 95%CI: 0.010-0-030).

494

495 The concentration of the less virulent strain in monocultures at the low nutrient level
496 was lower than the concentration of the virulent strain during the first three days (Fig
497 3C). This is indicated by negative posterior mean -5.479 and 95% CI not overlapping
498 zero (-9.029 – -1.662) for less virulent strain: low nutrient level interaction effect. The
499 concentration of the co-culture did not differ from the concentration of the virulent
500 strain monoculture at low nutrient level (posterior mean 0.722; 95% CI: -3.067 –
501 4.257 for co-culture: low nutrient interaction; Table S5). However, after 6 days the
502 monocultures of the less virulent strain at low nutrient level reached almost the same
503 concentration as the virulent strain monocultures and the co-culture (Fig. 3C), as
504 suggested by a significant positive deviance from zero for the posterior mean for the
505 3-way interaction between less virulent strain: low nutrient level: day (0.353, 95%CI:
506 -0.007 –0.670; Table S5, Fig. 3C). At high nutrient level the growth of the less
507 virulent strain monocultures, the virulent strain monocultures and the co-cultures did
508 not differ from each other (95%CI of less virulent strain B398 and co-culture
509 overlapped zero; Table S5, Fig. 3D).

510

511 The initial population growth during the first 24 h differed between the strains in the
512 low and high nutrient levels in the monocultures (ANOVA: strain $F_{1,6} = 88.258$, $p< 0.001$,
513 nutrient level $F_{1,6} = 279.167$, $p< 0.001$, nutrient level x strain effect $F_{1,6} =$
514 87.872, $p< 0.001$), but to opposite direction as compared to the low resource renewal
515 experiment. The virulent and the less virulent strain had similar initial growth in the
516 high nutrient level, but in the low nutrient level the initial growth was higher for the
517 virulent strain than for the less virulent strain (pairwise comparisons, Table S7). The
518 initial population growth was higher at high nutrient level than under low nutrient
519 level for both strains (Table S7).

520

521 In the second part of the experiment testing whether adaptation to a particular
522 resource level in monocultures changed the competition outcome (Fig. 2, 2. co-
523 culture), the proportion of the virulent strain in the competition treatment increased
524 during the experiment (MCMCglmm model: posterior mean for day 0.029, 95% CI:
525 0.021 – 0.037, Table S6, Fig. 2C). However, the two chemostat (low nutrient level)
526 co-cultures behaved very differently, thus nutrient level was not included in the
527 statistical analysis. The inoculations taken from the monocultures at day 24 did not

528 result in equal proportion of the less virulent and virulent strains in co-cultures, but
529 the proportion of the virulent strain was lower, ca. 30 % for the high resource
530 treatment and the other one of the chemostats and only a few percent for the other
531 chemostat. In spite of lower proportion at the start, and remaining at very low level
532 for 7-10 days in the high nutrient level co-cultures and the other chemostat, the
533 virulent strain eventually outcompeted the less virulent strain at both nutrient levels
534 (Fig. 2C).
535
536 The concentrations of the virulent and less virulent strain monocultures in the second
537 part of the high rate resource renewal experiment (Fig. 3, 2. co-culture) were higher
538 than those of the co-cultures, because the monocultures were continuously maintained
539 since the beginning of the first part and co-cultures were initiated with a lower
540 concentration (MCMCglmm model: posterior mean -3.645, 95% CI: -5.020 – -2.228
541 for co-culture). However, the concentration of the co-culture increased during the
542 experiment (co-culture:day -0.170, 95% CI: 0.098 – 0.239; Fig. 3C-D). The bacterial
543 concentrations were lower at low nutrient level (posterior mean -1.640, 95% CI: -
544 3.013 – -0.153) and they decreased during the experiment as compared to the high
545 nutrient level (posterior mean -0.239, 95% CI: -0.293 – -0.179 for low nutrient
546 level:day interaction, Table S6, Fig. 3C-D). There was no difference in growth
547 between the less virulent and the virulent strain (95% CI for the posterior mean for
548 B398 less virulent strain overlapped zero, Table S6).
549

550 **Biofilm formation**
551

552 In the experiment evaluating differences in relative amount of biofilm formation
553 between the virulent and less virulent strain in the high rate resource renewal
554 experiment, the less virulent strain B398 formed more biofilm than the virulent strain
555 B402 (ANOVA: $F_{1,23} = 6.65$, p=0.02). There was no interaction between the strain
556 and resource level ($F_{1,23} = 2.33$, p=0.14).
557

558 **Interference competition**
559

560 In the experiment evaluating the potential for interference competition via excreted
561 products between the strains, the virulent strain B402 inhibited the growth of the less
562 virulent strain B407 used in the batch experiment in all 3 replicates, but no inhibition
563 was observed vice versa. There was no inhibition between the virulent strain B402
564 and the less virulent strain B398 used in the high rate resource renewal experiment.
565

566 **Discussion**
567

568 Both the within host and the outside host environment affect the ecology and
569 evolution of environmentally growing opportunistic pathogens (Brown, et al. 2012).
570 The two environments may select for different properties for example in resource use,
571 but on the other hand growth in one environment may also pre-adapt for utilisation of
572 resources in the other environment (Ketola, et al. 2016, New, et al. 2014).
573 Environmental resources may thus affect pathogen virulence by inducing changes in
574 host utilisation rates. Here we tested the hypothesis that higher nutrient availability in
575 the outside host environment increases the virulence of an environmentally growing
576 opportunistic pathogen of fish, *Flavobacterium columnare*. We expected that
577 evolving in high nutrient environment would increase virulence due to selection for
578 fast resource use. As virulent strains of *F .columnare* have higher growth rate than
579 less virulent strains (Pulkkinen, et al. 2010), we also expected that high nutrient
580 conditions would favour virulent strains in competition.
581

582 Increased resource availability in the outside-host environment could increase
583 virulence in *F. columnare* by increasing bacterial population size, and thus the dose
584 encountered by the host (Kinnula, et al. 2017b, Kinnula, et al. 2015), or by facilitating
585 host invasion via virulence factor activation (Kinnula, et al. 2017b, Penttinen, et al.
586 2016), such as increased expression of tissue-degrading enzymes (Penttinen, et al.
587 2016). In the current experiments, however, immediate nutrient environment did not
588 affect the virulence, as we sub-cultivated all bacteria in 100% Shieh medium and
589 adjusted the dose prior the fish challenge. Therefore any changes in virulence were
590 expected to be due to adaptation in the nutrient environment where the strain was
591 evolving. In contrast to our expectations, we did not detect increase in virulence after
592 evolving in the high nutrient level for either the virulent or the less virulent strains as

593 compared to ancestors. Instead, the virulent strain maintained its virulence at the same
594 high level independent of the nutrient level and resource renewal rate. These results
595 suggest that increased resources in the outside-host environment do not affect
596 virulence in highly virulent strains. However, the less virulent strain decreased in
597 virulence in cultivation at constant low nutrient supply in chemostats or in long serial
598 cultivation in tubes in the high rate resource renewal experiment. Loss of virulence
599 has been commonly observed in pathogenic micro-organisms upon successive sub-
600 cultivation *in vitro* (Ford, et al. 2002, Gonzalez-Carrillo, et al. 2016, Moody, et al.
601 1990, Songe, et al. 2014), pointing to trade-offs between long-term survival in
602 outside-host and within-host environment (Ferenci 2016) or relaxed selection for
603 virulence in the outside-host environment (Mikonranta, et al. 2012). Increased supply
604 of resources might therefore maintain virulence at higher level in less virulent strains
605 in the outside-host environment.
606
607 In *F. columnare,* virulence is associated with rhizoid colony morphology, with a loss
608 of virulence upon change into two other morphs (rough or soft) (Kunttu, et al. 2011,
609 Kunttu, et al. 2009a). Maintenance of *F. columnare* for 5 months in stationary
610 starvation conditions led to diversification in colony morphology and virulence such
611 that rhizoid morph became more virulent and rough morph less virulent than the
612 ancestral strain (Sundberg, et al. 2014). The strains used in the current experiment
613 expressed originally rhizoid colonies upon cultivation on agar plates and no change
614 was observed during cultivation in batches. Bacteria from chemostats, however,
615 formed colonies that were intermediate between rhizoid and soft morphs
616 (Supplementary Fig. S5). The appearance of cells giving rise to soft colonies might
617 thus have decreased the overall virulence of the chemostat population. Resources in
618 the outside-host environment might contribute to the virulence of *F. columnare* via
619 diversification of cell types forming different colony morphologies, which might be
620 related to trade-offs in acquisition of nutrients in outside-host and within-host
621 environments. The mechanisms behind the different colony morphologies and their
622 virulence in *F. columnare* are not clear, but virulence is associated with gliding
623 motility of the cells, which in turn is affected by nutrient availability (Laanto, et al.
624 2014).
625

626 In direct competition over resources, the competitor with the fastest turnover rate of
627 resources into biomass is expected to win. In *F. columnare*, individually assessed
628 growth rates have been found to have a good agreement with the outcome of
629 competition in liquid culture (Ashrafi, et al. 2017). However, in current experiments,
630 outcome of competition in co-culture could be explained only in two cases with
631 differences in the initial growth rates of the strains measured in monocultures at
632 respective nutrient levels. Instead, we found that the virulent strain was superior in
633 competition both at high and low nutrient level regardless of the resource renewal
634 rate. The virulent strain outcompeted the less virulent strains in co-cultures in a few
635 days even after adaptation to prevailing nutrient level and after starting from a very
636 low proportion (see Fig. 2C). Similar genotype based dominance has been detected
637 previously (Kinnula, et al. 2017a) and more studies are needed to clarify whether the
638 traits harboured by the dominant strains are common to all virulent genotypes of *F.*
639 *columnare*.
640
641 Apart from direct competition over resources, microbes compete with each other with
642 various indirect mechanisms, including chemical interference (Hibbing, et al. 2010).
643 For *F. columnare*, interference competition is common (Ashrafi, et al. 2017, Kinnula,
644 et al. 2017a, Sundberg, et al. 2016). Here we confirmed growth inhibition of the less
645 virulent strain B407 by the virulent strain B402, which might explain the competition
646 outcome in the batch experiment. However, the competitive interactions via toxin
647 production have been detected for colonies growing on surfaces, and it is not clear
648 whether they play a role for planktonic growth (Cornforth and Foster 2013). The pair
649 used in the high rate resource renewal experiment did not inhibit each other's growth,
650 offering no explanation for the competition outcome in this experiment.
651
652 In addition to resource level, resource renewal rate can affect different traits in growth
653 (Finkel 2006, Velicer and Lenski 1999). Even though we expected that bacteria
654 grown under high nutrient level would be selected for fast growth (Frank 2010,
655 Litchman, et al. 2015, Velicer and Lenski 1999), it is possible that conditions in the
656 batches with low rate renewal of resources selected similar traits under both low and
657 high nutrient level. Without continuous renewal of nutrients, the bacteria might have
658 been adapted for fast growth before reaching carrying capacity well prior to the next
659 resource renewal. This saturation phase might then select for survival of bacterial

660 cells that are capable of remaining viable in starvation conditions (Arias, et al. 2012)
661 or capable of saprophytic usage of their conspecific cells. *F. columnare* has been
662 shown to survive and remain infective for several months when maintained in water
663 without added nutrients (Arias, et al. 2012, Kunttu, et al. 2009b, Sundberg, et al.
664 2016), and this feature has been attributed to a saprophytic capacity in the bacterium,
665 supported by experiments showing replication in fish carcasses (Kunttu, et al. 2009b),
666 (but see (Arias, et al. 2012) for an opposing view). It is also possible that nutrients
667 were depleted in the tube cultures with high rate resource renewal despite of the high
668 dilution aimed at maintaining the cultures at exponential growth stage prior daily
669 serial culture, and then these conditions might have selected for survival rather than
670 for high growth rate as well (Velicer and Lenski 1999).
671
672 On the other hand, the conditions in flow-through chemostats, favouring cells that can
673 grow and multiply at lowest nutrient level, were expected to favour the less virulent
674 strain (Gresham and Hong 2015, Litchman, et al. 2015, Velicer and Lenski 1999). In
675 the high rate resource renewal experiment, the less virulent strain had a propensity to
676 form biofilm on chemostat walls, and its higher relative biofilm forming capacity as
677 compared to the virulent strain was confirmed in a separate experiment on 96-well
678 plates at both nutrient levels. As no inhibition was detected between the pair used in
679 this experiment, the competition outcome in chemostats was possibly driven by the
680 less virulent strain diverting part of the population growth into the biofilm instead of
681 liquid culture. In high nutrient level, however, the daily transfer might have prevented
682 formation of biofilm for the less virulent strain, as no differences in concentration
683 between strains were seen. However, adhesion capacity has not been found to be
684 connected with virulence in *F. columnare* (Kunttu, et al. 2009a, Suomalainen, et al.
685 2006b). In fish farming conditions, tendency to form biofilm could give at least short-
686 term competitive advantage or a possibility to survive also for less virulent strains in
687 situations where the entire water mass of a fish tank is renewed.
688
689 In addition to fish farms, *F. columnare* can be commonly isolated from lake water and
690 biofilms at shore (Kunttu, et al. 2012). The selection regime for survival and
691 infectivity might greatly differ between these two environments. In the lake water the
692 resource level is still much lower than the lowest resource level used in this study (2%
693 Shieh medium). In a fish farming environment, excess nutrients are released to the

694 water from fish excretion, faeces and uneaten feed in varying concentrations
695 depending for example on the fish species, biomass, water exchange rate, feeding rate
696 and temperature (Lalonde, et al. 2015). Thus the resource quality available in the
697 outside host environment matches closely the resources provided by the host and
698 could pre-adapt the bacteria for faster host exploitation. Our results suggest that the
699 outside-host nutrient environment has less impact on strains that are highly virulent,
700 but rich resources in the outside-host environment might have significance in
701 maintaining virulence in less virulent strains and increase the contribution of less
702 virulent strains in columnaris epidemics in aquaculture. As inter-strain interactions of
703 the co-infecting strains might determine the outcome of infection (Kinnula, et al.
704 2017a), further studies are needed on the role of outside-host resources in maintaining
705 strain diversity and driving evolution of virulence among environmentally growing
706 pathogens.
707

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709

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715

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719

720 **References**
721

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Yoshida T, Jones LE, Ellner SP *et al.* Rapid evolution drives ecological dynamics in a predator-prey system. *Nature* 2003;**424**: 303-6.

900 Table 1. Model selection and the summary of the best fitting model on the
901 morbidity risk of the rainbow trout in the fish challenge experiment testing the
902 strains used in the batch experiment. Model selection is based on Akaike
903 information criteria (AIC). The best fit model estimating morbidity risk of the
904 host (rainbow trout) within time is marked with bold. P value indicates the
905 significance of the term removed from the higher model based on log-likelihood
906 test (LRT).
907

Model [a]	AIC	DF	LRT	p
S+T+W+S:T+S:W+T:W+S:T:W	302.65	128		
S+T+W+S:T+S:W+T:W	298.91	130	0.255	0.88
S+T+W+S:T+T:W	297.08	131	0.178	0.67
S+T+W+S:W+T:W	295.28	132	0.374	0.83
S+T+W+S:T+S:W	294.93	132	0.026	0.99
S+T+W+S:T	293.10	133	0.170	0.68
S+T+W+S:W	291.30	134	0.832	0.83
S+W+S:W	288.45	136	1.149	0.56
S+T+W	289.43	135	0.130	0.72
S+W	286.65	137	1.218	0.54
W	293.28	138	0.943	0.33
S	**285.63**	**138**	**8.626**	**0.003**
Summary of the best fitting model				
Source	**Estimate**	**SE**	**z value**	**p**
(Intercept)[b]	-2.9	0.13	-23.607	< 0.001
Strain(Less virulent)	-0.53	0.18	-2.988	0.003

908 [a] S, strain (virulent B402, less virulent B407); T, treatment (ancestor, low
909 nutrient level, high nutrient level), W, fish weight; +, main effect; colon,
910 interaction. [b] Intercept includes the effect of the virulent strain.
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924 Table 2. Model selection, summary of the best fitting model and the significance
925 of the variables included in the best fitting model on the morbidity risk of the
926 rainbow trout in the fish challenge experiment testing the strains used in the high
927 rate resource renewal experiment. Model selection is based on Akaike
928 information criteria (AIC). The best fit model estimating morbidity risk of the
929 host (rainbow trout) within time is marked with bold. P value indicates the
930 significance of the term removed from the higher model based on log-likelihood
931 test (LRT).
932

Model[a]	**AIC**	**DF**	**LRT**	**p**
S+T+W+S:T+S:W+T:W+S:T:W	424.52	200		
S+T+W+S:T+S:W+T:W	419.59	204	3.064	0.547
S+T+W+S:T+S:W	412.58	208	0.991	0.911
S+T+W+S:T	411.49	209	0.913	0.339
S+T+S:T	**410.97**	**210**	**8.947**	**0.062**
Summary of the best fitting model				
Source	**Estimate**	**SE**	**z value**	**p**
(Intercept)[b]	-3.17	0.32	-9.805	**< 0.001**
Strain(Less virulent)	-0.50	0.47	-1.079	0.2808
High day 24	-0.04	0.37	-0.104	0.9175
High day 52	-0.01	0.37	-0.030	0.9761
Low day 24	0.03	0.40	0.076	0.9396
Low day 52	-0.10	0.40	-0.254	0.7996
Less virulent: high day 24	-0.69	0.57	-1.223	0.2212
Less virulent: high day 52	-1.39	0.32	-2.267	**0.0234**
Less virulent: low day 24	-1.82	0.72	-2.515	**0.0119**
Less virulent: low day 52	-1.11	0.64	-1.732	0.0833
Significance of variables included in the best fitting model				
Source	**df**	**Deviance**	**Residual deviance**	**p**
Null			182.32	
Bacterial strain	1	78.62	103.71	**<0.001**
Treatment	4	3.81	99.90	0.432
Strain:treatment	4	8.95	90.95	0.062

933 [a] S, strain (virulent B402, less virulent B407); T, treatment (ancestor, low
934 nutrient level day 24 sample, low nutrient level day 52 sample, high nutrient
935 level day 24 sample, high nutrient level day 52 sample), W, fish weight; +, main
936 effect; colon, interaction. [b] Intercept includes the effect of the ancestral virulent
937 strain.
938

Figure captions

Fig. 1 The predicted cumulative mortality of rainbow trout (*Oncorhynchus mykiss*) fingerlings in continuous challenge experiments with *Flavobacterium columnare*. A) In fish challenge with bacteria cultivated in monocultures in the batch experiment, the risk depended only on the strain identity of the bacterium, but not whether it was ancestral isolate or cultured under low or high resource level. B) In fish challenge with bacteria from the monocultures from the experiment with high rate resource renewal, culturing in different nutrient environments did not change the virulence in the virulent strain (high = high nutrient level, low = low nutrient level). The less virulent strain decreased in virulence after cultivation at low nutrient level and after the longer cultivation period (52 days) at high nutrient level.

Fig. 2 The proportion of the virulent strain in co-cultures (mean ± SE): A) in the experiment with low resource renewal, B) in the experiment with high resource renewal started from ancestral isolates (1. co-culture) and C) in the experiment with high resource renewal after starting the co-cultures with bacteria from the monocultures after adaptation to a particular nutrient level (2. co-culture). In panel C, the proportion of the virulent strain is shown separately for both low nutrient level replicates due to large differences between replicates. Note that due to technical reasons (see Supporting information), the calculated proportion of the virulent strain may slightly exceed 1. The two values below the x-axis in C) denote for the corresponding days for monocultures in comparison to Fig. 3C-D.

Fig. 3 The bacterial concentrations (CFU ml^{-1}) in monocultures of the less virulent strain (Less virulent), monocultures of the virulent strain (Virulent) and co-cultures (L+V). A) Batch experiment (low resource renewal) at nutrient levels 5% (low nutrient) and B) 50% Shieh medium (high nutrient). C) The experiment with high rate resource renewal at 2% Shieh medium (low nutrient) and D) 20% Shieh medium (high nutrient). In the beginning of the high rate resource renewal experiment the co-cultures were started from ancestral isolates and discarded at day 20 (1. co-culture). New co-cultures were started (2. co-culture) with bacteria from the monocultures after

adaptation to certain nutrient level. Monocultures were maintained continuously for the whole duration of the high rate resource renewal experiment.

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