Author(s): Sundberg, Lotta-Riina; Ketola, Tarmo; Laanto, Elina; Kinnula, Hanna; Bamford, Jaana; Penttinen, Reetta; Mappes, Johanna

Title: Intensive aquaculture selects for increased virulence and interference competition in bacteria

Year: 2016

Version:

Please cite the original version:

Sundberg, L.-R., Ketola, T., Laanto, E., Kinnula, H., Bamford, J., Penttinen, R., & Mappes, J. (2016). Intensive aquaculture selects for increased virulence and interference competition in bacteria. Proceedings of the Royal Society B: Biological Sciences, 283(1826), Article 20153069. https://doi.org/10.1098/rspb.2015.3069

Intensive aquaculture selects for increased virulence and interference competition in bacteria

Lotta-Riina Sundberg*, Tarmo Ketola, Elina Laanto, Hanna Kinnula, Jaana K.H. Bamford, Reetta Penttinen, and Johanna Mappes

University of Jyvaskyla, Center of Excellence in Biological Interactions, Department of Biological and Environmental Science (and Nanoscience Center), Po box 35, FI-40014 University of Jyvaskyla, Finland

Corresponding author: Lotta-Riina Sundberg, University of Jyvaskyla, Center of Excellence in Biological Interactions, Department of Biological and Environmental Science (and Nanoscience Center), Po box 35, FI-40014 University of Jyvaskyla, Finland
Tel. +358 40 8053931, email: lotta-riina.sundberg@jyu.fi

KEY WORDS: aquaculture, evolution, fish farming, Flavobacterium columnare, pathogen, virulence

Abstract

Although increased disease severity driven by intensive farming practices is problematic in food production, the role of evolutionary change in disease is not well understood in these environments. Experiments on parasite evolution are traditionally conducted using laboratory models, often unrelated to economically important systems. We compared how the virulence, growth and competitive ability of a globally important fish pathogen, *Flavobacterium columnare*, change under intensive aquaculture. We characterized bacterial isolates from disease outbreaks at fish farms during 2003-2010, and compared *F. columnare* populations in inlet water and outlet water of a fish farm during the 2010 outbreak. Our data suggest that the farming environment may select for bacterial strains that have high virulence at both long and short time scales, and it seems that these strains have also evolved increased ability for interference competition. Our results are consistent with the suggestion that selection pressures at fish farms can cause rapid changes in pathogen populations, which are likely to have long-lasting evolutionary effects on pathogen virulence. A better understanding of these evolutionary effects will be vital in prevention and control of disease outbreaks to secure food production.

Background

Human interventions in food production create environments where the ecology, epidemiology and evolution of disease differ from nature. Increased host density and decreased host diversity can reduce the costs of pathogen transmission, and, according to the predictions of virulence evolution theory, highly virulent strains may emergence as a consequence [1,2]. Indeed, intensive farming environments are considered evolutionary hotspots, where the enhanced transmission and frequency of infections could promote virulence evolution in pathogen populations [3-7]. This can be especially important for environmentally transmitted opportunistic pathogens that often have the ability to survive and replicate outside hosts. These opportunistic pathogens do not suffer from the transmission-virulence trade-off similarly to the obligate pathogens, that are dependent on host survival during transmission, which may significantly alter pathogen virulence [8].

It is common that hosts in nature are co-infected by several pathogen strains. The within-host competitive interactions have been traditionally linked to virulence via faster growth of more virulent parasite strains which is expected to influence dynamics of disease epidemics [1,2,9-14]. However, bacterial interactions towards each other are mainly negative [15]. Competition over limited resources can promote selective interference competition mechanisms targeted to exclude competitors through the release of toxins [16,17], and lead to decreased virulence during bacterial infection [18,19]. It is thus likely that the interactions between co-existing pathogen strains influence the development and persistence of disease epidemics, and the population dynamics of pathogens.

Although these abovementioned theories and laboratory studies have provided insight on pathogen evolution, very little is still known about how anthropogenic changes, like intensive farming, influence virulence evolution [20-22]. Such empirical evidence is crucial for testing the theoretical predictions, but also for understanding disease ecology and evolution. More than anything, we need data outside of the laboratory from the economically relevant systems [21]. Information on the diseases threatening crop, livestock and aquaculture production is essential for securing world food production. Here, we study how selection in intensive aquaculture influences the virulence and the competitive ability, at both long and short time scales, in the globally significant fish pathogen *Flavobacterium columnare* (Bacteroidetes) [23,24]. *First,* we explore temporal changes in virulence and competitive ability using *F. columnare* strains isolated from disease outbreaks in 2003-2010. *Second,* we study locally occurring selection by comparing bacterial isolates originating from inlet water and outlet water of a fish farm during one outbreak season in summer 2010. We observe that intensive aquaculture seems to select for increased *F. columnare* virulence, as well as ability for

interference competition, at both short and long timescales. This indicates that the more virulent strains have higher fitness under intensive farming conditions, and demonstrates that the intensive farming environments can be used as model systems to understand disease dynamics and evolution of virulence.

Methods

Experimental set-up

In this study we used two different sets of *F. columnare* strains to study evolution of bacterial virulence and competitive ability on both at temporal and spatial scale. In the first set, we used strains isolated during 2003 – 2010. In the second set we compared the characteristics of bacteria isolated in 2010 from the inlet and the outlet water of a fish farm. In both study settings we studied the bacterial growth rate, the bacterial virulence in zebra fish, and the ability of the bacteria to inhibit the growth of other strains, and analysed if these traits changed in time and place. In addition, the data from 2003-2010 was used to analyse the bacterial competition in finite resources in liquid culture, and the growth of the inlet water and outlet water strains were compared in two different resource concentrations.

Bacterial strains, their isolation and genetic characterization

The bacteria were isolated originally from three different fish farms in Central and Northern Finland during columnaris disease epidemics and from environment in 2003-2010 (Table 1) using standard culture methods on Shieh medium [25], [26] or AO-agar [27]. Isolates for the dataset were randomly chosen from a large collection of uncharacterized bacterial strains without any a priori information on their competitive ability or virulence, except for strains H2, B067, B185, B245, B405 and B407 that have been used (separately) in our previous studies [28-30].

The bacterial strains from inlet water upstream of a fish farm [29] and from downstream and outlet water of the same farm were collected in summer 2010 (Table 2). Both locations (inlet water and outlet water) were sampled on 8th and 21st June, on 5th, 12th, 19th and 26th July, and on 2nd, 16th and 30th August in 2010 (Supplementary Table 1). Nine bacterial isolates were randomly selected from both locations for further analyses. After isolation, the bacteria were maintained frozen in -80°C with 10% glycerol and 10% fetal calf serum. For the analyses, the bacterial strains were grown in Shieh medium at room temperature (RT, approximately 24ºC) under constant shaking (110 rpm) 24-48h and enriched overnight.

The strains collected in 2003-2010 and the inlet water strains were genetically characterized in an earlier study using Multilocus Sequence Analysis (MLSA) [31] and Automated Ribosomal Intergenic Spacer Analysis (ARISA) [29]. The MLSA method produces genetic clustering comparable to the ARISA method [31] and the two methods can be used interchangeably. The outlet water strains and one inlet water strain (B355), were genotyped in this study using ARISA, as described earlier [29,32] (see Supplementary Material for more details). The possible presence of plasmids in the strains was studied from 3.8 ml of overnight grown turbid cultures using a QIAprep® Spin Miniprep kit (Qiagen) following manufacturer's instructions. *Flavobacterium* sp. strain B330 harbouring a natural plasmid was used as a positive control. DNA was run in 0.8 % agarose gel and visualized under UV light to detect the presence/absence of plasmids.

Interference competition assays

The inhibitory activity of *F. columnare* strains was studied both in time (17 strains isolated in 2003-2010) and space (nine inlet water strains vs. nine outlet water strains). Within each set of strains, inhibition was tested reciprocally using a double layer method and the assays were replicated three (strains from 2003-2010) or four (inlet vs. outlet water strains from 2010) times. The optical density (OD, at 570 nm) of the bacterial cultures was measured with spectrophotometer and adjusted between 0.250 and 0.290. Three hundred µl of fresh overnight-grown "recipient" bacterial culture was mixed with 3 ml of soft Shieh agar (0.7 %) tempered to 47 °C and poured on Shieh agar plates. Aliquot of the bacterial culture was centrifuged at 17 000 x G for 3 minutes in RT. Five microliters of the supernatant of the "donor" cultures were spotted on the surface of the top agar. Following an incubation of 48h at RT, the plates were checked to see whether the "donor" strain had caused a growth inhibition of the underlying "recipient" bacterial lawn. The inhibition was ranked as 0 = no inhibition, 1 = inhibition.

The interaction between *F. columnare* strains B067 and B185 was further characterized with filtered supernatant (0.2 µm PES filter, VWR) and by cross streaking on agar plates in 10 replicates. In the first experiment, turbid overnight grown liquid culture and filtered supernatant of strain B185 were cross streaked with B067 by using 1 µl loop. In the second experiment, 300 µl of turbid liquid culture of B067 was mixed with Shieh soft agar and plated, and 10 µl of B185 culture and filtered supernatant was applied on the soft agar. Growth inhibition was monitored after 48 h incubation.

Bacterial growth measurements

A temperature-controlled spectrophotometer (Bioscreen C®, Growth curves Ltd, Helsinki, Finland) was used to monitor the growth of the bacterial strains. Before the growth measurements of the both study sets, the optical densities of the fresh overnight-grown bacterial cultures were adjusted to 0.10-0.20 in A570 to minimize the differences in the initial turbidity between strains. Forty µl of each bacterial strain was inoculated onto 400 µl of sterile Shieh culture media on a BioScreen Honeycomb plate (100-well-plate, Oy Growth Curves Ab Ltd) in five replicates per strain, both in normal and diluted medium. The growth data was measured at 25 °C for 96 h at 5 min intervals (absorbance at 420–580 nm, wide band option).

The growth parameters were calculated from the raw data by utilizing matlab script written by TK in which the maximal growth rate is found from $\log_2$-transformed data by fitting linear regressions on 25 time-point sliding windows. The highest linear (log transformation linearizes the exponential growth) slope found in sliding windows equals the maximal growth. The yield is found as a maximal average optical density over 25 time point's sliding window in the raw data. The area under curve (AUC) sums the OD data over the entire measurement period to indicate the cumulative amount of biomass attained during the time.

Bacterial competition in liquid culture

We also studied competition in liquid culture (in finite resources) using the strains isolated in 2003-2010. Cultures and measurements were done similarly as above with the single strains (see above), but with 1:1 mixture of bacterial strain pairs (compared to individual bacterial strain bacteria diluted 1:1 with dH_20) and OD at 600 nm was measured every five minutes for 95 hours. Area under curve (AUC), a measure that describes the cumulating amount of biomass a given strain and strain-strain combinations can produce within a given time, gave roughly the same results as the yield. From the obtained AUC we calculated interaction indexes for the bacterial strains as

$$interaction\ index = \frac{growth(strain\ 1\ and\ 2\ together)}{growth\ (strain\ 1\ alone) + growth\ (strain\ 2\ alone)}$$

For the data analysis, the interaction index was arcsin-transformed (see data analysis below).

Virulence in zebra fish

To measure the virulence of the studied bacterial strains, unsexed, adult, disease-free zebra fish were obtained from Core Facilities (COFA) and research services of Tampere (Tampere University, Finland). The zebra fish response to *F. columnare* infection is qualitatively similar to the common host of the pathogen in aquaculture, rainbow trout [33]. The optical density of the overnight-grown bacterial culture (570 nm) was measured and the corresponding bacterial density in colony forming units (cfu) was calculated according to our previously fitted standard curve. The infection method and bacterial dose used were optimized in preliminary experiments.

The bacterial exposure with nine inlet water strains and nine outlet water strains was done using 14 replicate fish per strain. In addition, 14 control fish received sterile growth medium and served as a negative control group. In total, 266 individual zebra fish were used in the experiment. Five ml of sterile Shieh culture medium mixed with bacterial culture (pure culture medium in the negative control) was pipetted directly into each aquarium to reach the infective dose of 1×10^4 colony forming units (cfu) ml^{-1} in the water throughout the experiment, as a continuous exposure. The fish were then monitored for 11 d for disease symptoms and morbidity. For the first 3 days, during the most acute phase of the disease, the fish were monitored every hour and when the progression of the epidemic ceased the monitoring points were decreased accordingly, including at least two checks per day.

The virulence of strains isolated in 2003-2010 was tested similarly but with small modifications. Infection with each bacterial strain (11 strains of the dataset) was done to 10 replicate fish. In addition, 10 fish in the negative control group were exposed to sterile culture medium, thus the total number of individual fish in this experiment was 120. In this experiment the bacteria were mixed in 550 µl of Shieh medium and pipetted into aquaria to reach a dose of 2.5×10^5 cfu ml^{-1}. The fish were monitored every hour for 40 hours.

During the experiments the fish were held in individual 750 ml plastic aquaria with 500 ml of ground water (average t=24.7°C). Morbid fish that had lost their natural swimming buoyancy and did not respond to external stimuli were considered dead and removed from the experiment. All the remaining healthy fish at the end of the experiment were euthanized by cutting the spinal cord under terminal aenesthesia with MS-222 (Sigma). All fish were weighted and a bacterial culture sample was taken from the gills on Shieh agar supplemented with tobramycin [26] to ensure the cause of death to be columnaris disease.

The experiments were conducted according to the Finnish Act on the Use of Animals for Experimental Purposes, under permission ESAVI-2010-05569/Ym-23 granted for L-RS by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

Data-analyses

The inhibition data of inlet water and outlet water strains was analysed using generalized linear mixed models implemented in function glm, in R. Inhibition data was modeled with quasipoisson distribution, explaining pooled replicates (within each reciprocal combination, considered as separate combination) with 4 levels fixed factor representing all combinations of receiver and donor being an inlet or outlet strains (without multiple comparisons). The inhibition data of strains isolated in 2003-2010 was analyzed with similar method from averaged data, but using year of isolation of donor and recipient strains as continuous covariates (both rank transformed). The replicates were pooled due to problems in mixed model fitting of random effects of strain combination that prevented accurate estimates of 2003-2010 data. The results obtained from mixed models, in both datasets, give equivalent biological interpretation of the data that is presented here.

In competition in liquid culture there are no producer or receiver strains that could be distinguished, and we could only test the effects of average isolation year and difference of the isolation years on the interaction index, in addition to the strain combination identity. The growth traits of all bacterial isolates, and the competition indices for time series 2003-2010, were analysed with mixed models in SPSS.

Virulence of bacterial strains in both of the datasets (time series and the inlet vs outlet water strains) was first analysed by Kaplan Meier survival analysis (log rank Mantel cox). The fish alive at the end of experiments were treated as censored data. In addition to the log rank analysis, we also used average longevity as a measure of virulence with strains isolated in 2003-2010. However, in the dataset of the inlet and outlet water strains only 10% of the fish infected with the inlet water strains (and 40 % infected with the outlet water strains) entered a moribund state during the experiment, precluding the use of the fish longevity as a surrogate of virulence. Therefore, for the inlet and outlet water strains, we used the log rank Mantel cox survival analysis, and also arcsin-square transformed mortality percentage as a measure of virulence. The mortality percentages of fish were tested with t-tests allowing for the unequal variances [34].

Results

Temporal evolution in *F. columnare* strains isolated in 2003-2010

Inhibitory toxin production, competitive ability and virulence were significantly associated with the time of isolation in strains from different fish farms and environmental locations (2003-2010). The isolation year had a significant effect on bacterial virulence (log rank Mantel cox, χ^2= 55.338, df=3, p<0.001). The more recently isolated bacteria were significantly more virulent (average longevity of the infected fish) than the ones isolated earlier (b=-1.716, p=0.014) (Figure 1a). In line with the virulence, the more recently isolated strains were more likely to inhibit the earlier isolates (b=0.004, p=0.0181). The sensitivity of the bacteria to the interference by the other strains was independent of the isolation time (year (rank-transformed) of isolation of the inhibited isolate b=0.002, p=0.2975) (see Supplementary Table 2a for original data). The year of isolation did not have an effect on the bacterial growth parameters (maximum growth rate, yield or area under curve AUC) whereas the strain identity did (Supplementary Table 3).

To elaborate the benefits of interference in resource competition, we studied the intensity of competition between the isolates in co-culture, where the competition on finite resources is expected to lead to lower production of biomass if the competing strains use the same resource. The competition was more intense (indicated by lower interaction index) between strains that on average had been isolated more recently, than between the strains that were isolated earlier (Table 3, Figure 2). The difference in the isolation times of the competing strains did not significantly affect the interaction index (Table 3).

The cross-streaking experiments of the strains B185 and B067 indicated that toxin production in *F. columnare* could be contact-dependent. When cross-streaked on agar-plate, in nine out of ten replicates clear inhibitory zones (similar to Dienes lines) [35,36] were observed (Supplementary Figure 1), but only when supernatant containing bacterial cells was used. Sterile filtered supernatant did not cause any inhibition in any of the experiments.

Local, short timescale evolution

The farming environment was observed to have a significant impact on the population structure of the bacterial populations occurring in the inlet and outlet water. The bacterial population from the outlet water of the fish farm was genetically more homogenous than the population from the inlet

277 water upstream of the farm (Table 2). Some of the genotypes in the inlet water were not detected in
278 outlet water, indicating that the fish farming environment may select for specific genotypes.
279
280 Place of isolation (inlet or outlet water of a fish farm) had a significant effect on bacterial virulence
281 (log rank Mantel cox, χ^2= 33.471, df=2, p<0.001). Mean percentage of mortality in fish infected with
282 the outlet water isolates was 32.5%, and 10.3% with the inlet water strains (t=3.155, p=0.006, on
283 arcsin square transformed proportion of the dead fish) (Figure 1b). No background mortality in the
284 fish exposed to sterile growth medium (negative control group) was observed.
285
286 When the outlet water isolates acted as donors in an inhibition test against the inlet water isolates, the
287 risk of inhibition (0.225) seemed to be higher (z=-2.154, p=0.033) than when the outlet water strains
288 were let to inhibit other outlet water strains (0.167). Other types of inhibition pairs produced
289 intermediate inhibition risks and did not statistically differ from the other pairs (Table 4 and
290 Supplementary Table 2b). Since level of significance (p=0.033) of the only significant pairwise
291 comparison is not dramatically different from 0.05, there is a possibility that significance is
292 overstated without corrections for multiple testing, leading to acceptance error (type I error).
293 However, several papers suggest that multiple corrections could very easily lead to rejection errors
294 (type II), especially with small datasets, as here [37]. Hence, as we have not adopted corrections the
295 obtained result should be considered tentative.
296
297 To examine the effects resource concentrations on bacterial growth we studied the growth of the inlet
298 and the outlet water bacterial strains in two resource concentrations: in the standard growth medium
299 and in the diluted (0.5x) medium. In yield and AUC the outlet water strains (yield: 0.853, se: 0.052;
300 AUC: 647.6, se: 40.98) excelled over the inlet water strains (yield: 0.668, se: 0.052; AUC: 486.6, se:
301 40.98), but the growth rates of the two groups were comparable (inlet water: 0.216, se: 0.012; outlet
302 water: 0.199, se: 0.012) (Table 5, Supplementary Figure 2). Interestingly, though, the growth of the
303 outlet water strains was more sensitive to the differences in resource concentrations (low resource:
304 0.176, se: 0.013; high resource: 0.222, se: 0.013, Table 5) than the growth of the inlet water strains
305 (low resource: 0.208, se: 0.013; high resource: 0.225, se: 0.013).
306
307 Fifteen bacterial strains (including strains from both datasets) were screened for presence of
308 plasmids, but none were observed (Supplementary Figure 3).
309
310 **Discussion**

311 Since the mid-1990's, *F. columnare* outbreak frequency, severity of symptoms and disease-related
312 mortality have significantly increased in aquaculture, and the evolution of virulence has been
313 suggested as an explanation [23]. Our study finds that the bacterial strains isolated most recently,
314 during the period of more difficult disease outbreaks, are more virulent and have a higher
315 competitive ability than the strains isolated earlier. In addition, our findings about the *F. columnare*
316 populations from the inlet and outlet water of a fish farm are congruent with the suggestion of
317 selection of more virulent strains in aquaculture. The strains isolated from outlet water were more
318 virulent and able to produce higher maximum population sizes than the inlet water strains, indicating
319 that the bacteria with the best ability to exploit fish populations benefit the most in the intensive
320 aquaculture conditions.
321
322 Opportunistic lifestyle and ability to persist outside host opens less stringent trajectories for virulence
323 evolution, in contrast to the obligate pathogens restricted by the transmission-virulence trade-off [8],
324 but factors that increase or limit virulence evolution in opportunistic pathogens are still poorly
325 understood. *F. columnare* can be considered an opportunist, as it is known to survive long periods
326 outside the host and transmit efficiently from dead hosts [38-40]. A weak trade-off between
327 virulence and other fitness traits, like transmission, provided by the outside-host survival could thus
328 promote the evolution of high virulence in *F. columnare*. Moreover, in aquaculture, new susceptible
329 fish populations are introduced at the farm annually, creating conditions similar to serial passage
330 which further decreases the costs of virulence [9].
331
332 In addition to the factors related to host abundance, the bacterial communities in aquaculture are
333 shaped by increased concentrations of nutrients and chemical and antibiotic treatments within the
334 rearing units. Eutrophication of the aquatic environment increases parasitic and bacterial diseases via
335 direct and indirect effects in the food web [41,42]. While the role of eutrophication on *F. columnare*
336 epidemics is still unclear, the increased nutrient concentrations may support the outside-host growth
337 of this pathogen, giving the more virulent strains a greater advantage (Supplementary Figure 2), see
338 also [43]. On the other hand, the use of chemotherapy can relax the competitive interactions between
339 bacteria by eliminating sensitive species. This may lead to more frequent or virulent outbreaks of
340 opportunistic diseases if the use of antibiotics increases the intensity of the within-species
341 competition [44] or selects for faster transmission rate [45]. The increasing amount of antibiotics
342 used in food production [46] may thus affect microbial communities beyond the traditionally
343 expected environmental effects [47-49]. Therefore, to secure the global food production it is vital to
344 understand the factors that select for virulent pathogen strains.

On top of intensive farming practices *per se*, also other ecological and evolutionary factors underpin the evolution of more virulent and competitive bacteria at long timescales. A general increasing trend in disease species diversity and outbreak frequency has been observed during last decades, but the reasons for this are still largely unknown [50]. One contributing factor is the warming climate, that causes changes in disease ecology, outbreak dynamics and seasonality [51-53]. Due to the global warming, the longer outbreak period for columnaris disease [23] increases the bacterium-host and bacterium-bacterium interactions, allowing greater opportunities for evolution of both virulence and competitive ability.

Strain-strain interactions can have a significant role in bacterial disease dynamics via competition in both within-host and outside-host environments [54-58], and the surrounding microbial community has been shown to have significant effects on the evolution of interference [59,60]. However, how interference competition is associated with intensive farming is not properly understood. Our data suggest that the most recently isolated bacteria seem to have the highest capacity for interference competition and, on average, the competition between the most recently isolated strains is the most intense. Although the strains isolated from the inlet water were able to inhibit each other and the outlet water strains, significant differences in inhibition was observed only when the outlet water isolates acted as inhibitors. The strains isolated from outlet water seemed to be able to inhibit the growth of the inlet water strains but tolerated well the toxins produced by other outlet water strains, most likely due to more homogeneous population structure resulting in less competitive interactions [61]. While these results are in accordance with the general expectations of the evolution of interference competition in mixed populations [54,57], whether these interactions are relevant during disease outbreaks is unknown, and type I error in the interpretation of the results is possible. Nevertheless, different bacterial population structure was observed in these two locations even during the same sampling dates that are directly comparable (Table 2). These results suggest that the competition pressures differ within and outside the farming environment, and that the interference, as well as toxin tolerance, could be beneficial for the virulent strains in the farming environment where invasions by multiple strains are frequent.

Although this study does not aim to characterize the mechanism of growth inhibition, bacteriocins have been reported previously in *F. columnare* [62]. Our data, however, indicate that the toxin production in *F. columnare* may require a direct contact between bacterial cells (see Supplementary Figure 1), but more studies are needed to identify the cell-cell interactions in this species in detail.

Previous experimental studies have often demonstrated a direct trade-off between toxin production and both growth rate and virulence. Virulence of co-infection with a toxin-producing and a toxin-sensitive bacterial strain leads to a decrease in the total virulence of infection [18,19,63]. In contrast to traditional assumptions, our data show no evidence of costs in toxin production for growth (growth rate, population size) or virulence in *F. columnare*. Although the competitive ability and virulence increased in time, the bacteria isolated in 2003-2010 did not differ in their growth features. Similarly, the growth rate of the virulent bacteria isolated from the outlet water did not exceed the growth rate of the less competitive and low-virulence inlet water strains. However, the outlet water population reached higher population sizes regardless of the nutrient conditions. It seems that in this study system the same factors selecting for increased virulence might simultaneously also select for increased competitive ability. It remains unknown whether virulence and competitive ability are genetically linked and if their benefits are traded off with other life-history traits.

To conclude, in accordance with the theoretical predictions [1,7-9,21], our data are consistent with the hypothesis that the intensive farming conditions (high host densities, increased transmission opportunities, co-infections, possibility for serial passage, availability of nutrients, use of chemotherapy) can select for pathogen strains with the ability to produce large population sizes, high virulence that have high competitive ability under short time scales. This indicates a genetic difference in populations of high- and low-virulence bacterial strains resulting in selection for strains with an increased ability to exploit the fish host as a nutrient source. To reveal the possibility of horizontal gene transfer by conjugation as a mechanism for increased virulence, we assayed the presence of plasmids in the *F. columnare* bacterial strains, but similarly to previous studies [28] none were found. Therefore, other possible genetic mechanisms causing the changes in virulence (transduction, chromosomal transformation, mutations) [64] remain to be solved, and will require whole genome sequencing of several strains. Also the recent achievements in genetic manipulation techniques [65,66] and genome sequencing [67] are likely to provide detailed insight into the mechanisms behind *F. columnare* pathogenicity.

Our results indicate selection for pathogen virulence and competitive ability in both long and short time scales. The bacterial strains isolated in 2003 were significantly less virulent than the strains isolated later, which correlates with the observed increase in the severity of columnaris outbreaks during the last decade [23]. Interestingly, a similar pattern was observed already during one outbreak season, as the strains isolated from the outlet water of a fish farm were more virulent than strains

isolated from the inlet water. These results indicate that the selection pressures at fish farms can cause changes in pathogen populations, which may have long-lasting effects on pathogen virulence. The global changes in nutrients and climate can further select pathogen traits at a wider temporal scale. Indeed, we cannot rule out the possibility that the changes in bacterial characteristics observed in this paper are unrelated to aquaculture. Nevertheless, aquaculture has a major role in securing the world protein production, but disease epidemics severely affect the profitability of the industry [68-70]. Understanding the disease ecology and evolution in the man-made environments is important in securing sustainable livestock and aquaculture production. In addition to the benefits in the applied field, the studies on pathogens in the intensive farming systems can provide much needed empirical field data on the evolution of virulence.

Data accessibility

All data used in this paper are publicly available in supplementary file and in Dryad (doi:10.5061/dryad.nk76k).

Competing interests

Authors do not have competing interests.

Authors' contributions

L-RS, TK, JB and JM designed the study. L-RS, TK, EL, HK and RP collected and analysed the data. All authors participated in drafting and critically revised the manuscript. All authors approved the final version of the manuscript.

Acknowledgements

We would like to thank Dr Heidi Kunttu and Dr. Päivi Rintamäki for donating the bacterial isolates used in this study, Dr. Kunttu for help during the experiments, Dr Nina Pekkala, Mr Petri Papponen and MSc Katja Neuvonen for technical assistance in laboratory, and Dr Emily Burfield-Steel and Dr Andrés López-Sepulcre for helpful comments on the manuscript. This work was supported by the Finnish Centre of Excellence Program of the Academy of Finland, CoE in Biological Interactions 2012-2017 (#252411, J.M. and J.B.), by Academy of Finland grants #272995 (L.-R.S.) and #278751 (T.K.), and by Maj and Tor Nessling Foundation (J.B.).

447

448 **References**

449

450

451 1. Ebert, D. & Herre, E. 1996 The evolution of parasitic diseases. *Parasitol. Today* **12**, 96–101.

452 2. Frank, S. A. 1996 Models of parasite virulence. *Q. Rev. Biol.* **71**, 37–78.

453 3. Altizer, S., Harvell, D. & Friedle, E. 2003 Rapid evolutionary dynamics and disease threats to
454 biodiversity. *Trends in Ecology & Evolution* **18**, 589–596. (doi:10.1016/j.tree.2003.08.013)

455 4. Galvani, A. P. 2003 Epidemiology meets evolutionary ecology. *Trends Ecol. Evol.* **18**, 132–
456 139. (doi:10.1016/S0169-5347(02)00050-2)

457 5. Schrag, S. & Wiener, P. 2004 Emerging infectious disease: what are the relative roles of
458 ecology and evolution? *Trends Ecol. Evol.* **10**, 319–324.

459 6. Peeler, E. J. & Feist, S. W. 2011 Human intervention in freshwater ecosystems drives disease
460 emergence. *Freshwater Biol.* **56**, 705–716. (doi:10.1111/j.1365-2427.2011.02572.x)

461 7. Kennedy, D. A., Kurath, G., Brito, I. L., Purcell, M. K., Read, A. F., Winton, J. R. & Wargo,
462 A. R. 2015 Potential drivers of virulence evolution in aquaculture. *Evol. Appl.*, online early.
463 (doi:10.1111/eva.12342)

464 8. Brown, S. P., Cornforth, D. M. & Mideo, N. 2012 Evolution of virulence in opportunistic
465 pathogens: generalism, plasticity, and control. *Trends Microbiol.* **20**, 336–342.
466 (doi:10.1016/j.tim.2012.04.005)

467 9. Ebert, D. 1998 Experimental Evolution of Parasites. *Science* **282**, 1432–1436.
468 (doi:10.1126/science.282.5393.1432)

469 10. Read, A. F. & Taylor, L. H. 2001 The ecology of genetically diverse infections. *Science* **292**,
470 1099–1102.

471 11. Woolhouse, M. E. J., Webster, J. P., Domingo, E., Charlesworth, B. & Levin, B. R. 2002
472 Biological and biomedical implications of the co-evolution of pathogens and their hosts. *Nat.*
473 *Genet.* **32**, 569–577. (doi:10.1038/ng1202-569)

474 12. Karvonen, A., Rellstab, C., Louhi, K.-R. & Jokela, J. 2012 Synchronous attack is
475 advantageous: mixed genotype infections lead to higher infection success in trematode
476 parasites. *Proc. Royal Soc. B.* **279**, 171–176. (doi:10.1098/rspb.2011.0879)

477 13. Leggett, H. C., Benmayor, R., Hodgson, D. J. & Buckling, A. 2013 Experimental evolution of
478 adaptive phenotypic plasticity in a parasite. *Curr. Biol.* **23**, 139–142.
479 (doi:10.1016/j.cub.2012.11.045)

480 14. Susi, H., Barrès, B., Vale, P. F. & Laine, A.-L. 2015 Co-infection alters population dynamics
481 of infectious disease. *Nature Comm.* **6**, 5975. (doi:10.1038/ncomms6975)

482 15. Foster, K. R. & Bell, T. 2012 Competition, Not Cooperation, Dominates Interactions among
483 Culturable Microbial Species. *Curr. Biol.* **22**, 1845–1850. (doi:10.1016/j.cub.2012.08.005)

16. Brown, S. P., West, S. A., Diggle, S. P. & Griffin, A. S. 2009 Social evolution in micro-organisms and a Trojan horse approach to medical intervention strategies. *Phil. Trans. Royal Soc. B.* **364**, 3157–3168. (doi:10.1098/rstb.2009.0055)

17. Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. 2009 Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Micro.* **8**, 15–25. (doi:10.1038/nrmicro2259)

18. Massey, R. C., Buckling, A. & ffrench-Constant, R. 2004 Interference competition and parasite virulence. *Proc. Royal Soc. B.* **271**, 785–788. (doi:10.1098/rspb.2004.2676)

19. Inglis, R. F., Gardner, A., Cornelis, P. & Buckling, A. 2009 Spite and virulence in the bacterium Pseudomonas aeruginosa. *P. Natl. Acad. Sci.* **106**, 5703–5707. (doi:10.1073/pnas.0810850106)

20. Nowak, B. F. 2007 Parasitic diseases in marine cage culture – An example of experimental evolution of parasites? *Int. J. Parasitol.* **37**, 581–588. (doi:10.1016/j.ijpara.2007.01.003)

21. Mennerat, A., Nilsen, F., Ebert, D. & Skorping, A. 2010 Intensive Farming: Evolutionary Implications for Parasites and Pathogens. *Evol. Biol.* **37**, 59–67. (doi:10.1007/s11692-010-9089-0)

22. Kurath, G. & Winton, J. 2011 Complex dynamics at the interface between wild and domestic viruses of finfish. *Curr. Opin. Virol.* **1**, 73–80. (doi:10.1016/j.coviro.2011.05.010)

23. Pulkkinen, K., Suomalainen, L. R., Read, A. F., Ebert, D., Rintamaki, P. & Valtonen, E. T. 2010 Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. *Proc. Royal Soc. B.* **277**, 593–600. (doi:10.1098/rspb.2009.1659)

24. Declercq, A. M., Haesebrouck, F., Van den Broeck, W., Bossier, P. & Decostere, A. 2013 Columnaris disease in fish: a review with emphasis on bacterium-host interactions. *Vet. Res.* **44**, 27. (doi:10.1186/1297-9716-44-27)

25. Song, Y. L., Fryer, J. L. & Rohovec, J. S. 1988 Comparison of six media for the cultivation of Flexibacter columnaris. *Fish Pathol.* **23**, 91-94.

26. Decostere, A., Haesebrouck, F. & Devriese, L. 1997 Shieh medium supplemented with tobramycin for selective isolation of Flavobacterium columnare (Flexibacter columnaris) from diseased fish. *J. Clin. Microbiol.* **35**, 322–324.

27. Anacker, R. L. & Ordal, E. J. 1959 Studies on the Myxobacterium Chondrococcus columnaris: I. Serological Typing. *J. Bacteriol.* **78**, 25–32.

28. Suomalainen, L. R., Tiirola, M. & Valtonen, E. T. 2006 Chondroitin AC lyase activity is related to virulence of fish pathogenic Flavobacterium columnare. *J. Fish Dis.* **9**, 757–763.

29. Kunttu, H. M. T., Sundberg, L.-R., Pulkkinen, K. & Valtonen, E. T. 2012 Environment may be the source of Flavobacterium columnare outbreaks at fish farms. *Env. Microbiol. Rep.* **4**, 398–402. (doi:10.1111/j.1758-2229.2012.00342.x)

30. Laanto, E., Bamford, J. K. H., Laakso, J. & Sundberg, L.-R. 2012 Phage-driven loss of virulence in a fish pathogenic bacterium. *PLoS ONE* **7**, e53157.

(doi:10.1371/journal.pone.0053157)

31. Ashrafi, R., Pulkkinen, K., Sundberg, L.-R., Pekkala, N. & Ketola, T. 2015 A multilocus sequence analysis scheme for characterization of Flavobacterium columnare isolates. *BMC Microbiol* **15**, 245

32. Suomalainen, L. R., Kunttu, H., Valtonen, E. T., Hirvela-Koski, V. & Tiirola, M. 2006 Molecular diversity and growth features of Flavobacterium columnare strains isolated in Finland. *Dis Aquat Organ* **70**, 55–61.

33. Kinnula, H., Mappes, J., Valkonen, J. & Sundberg, L.-R. 2015 Influence of infection dose on the virulence of a generalist pathogen in a common and a novel host. *PLoS ONE* **10**(9), e0139378. http://doi.org/10.1371/journal.pone.0139378.t003

34. Ruxton, G. D. 2006 The unequal variance t-test is an underused alternative to Student's t-test and the Mann-Whitney U test. *Behav. Ecol.* **17**, 688–690. (doi:10.1093/beheco/ark016)

35. Gibbs, K. A., Urbanowski, M. L. & Greenberg, E. P. 2008 Genetic Determinants of Self Identity and Social Recognition in Bacteria. *Science* **321**, 256–259. (doi:10.1126/science.1160033)

36. Budding, A. E., Ingham, C. J., Bitter, W., Vandenbroucke-Grauls, C. M. & Schneeberger, P. M. 2009 The Dienes phenomenon: competition and territoriality in Swarming Proteus mirabilis. *J. Bacteriol.* **191**, 3892–3900. (doi:10.1128/JB.00975-08)

37. Moran D.M. 2003 Arguments for rejecting the sequential Bonferroni in ecological studies. *Oikos* **100**, 403-405.

38. Kunttu, H. M. T., Valtonen, E. T., Jokinen, E. I. & Suomalainen, L.-R. 2009 Saprophytism of a fish pathogen as a transmission strategy. *Epidemics* **1**, 96–100. (doi:10.1016/j.epidem.2009.04.003)

39. Arias, C. R., Lafrentz, S., Cai, W. & Olivares-Fuster, O. 2012 Adaptive response to starvation in the fish pathogen Flavobacterium columnare: cell viability and ultrastructural changes. *BMC Microbiol* **12**, 266. (doi:10.1186/1471-2180-12-266)

40. Sundberg, L.-R., Kunttu, H. M. T. & Valtonen, E. T. 2014 Starvation can diversify the population structure and virulence strategies of an environmentally transmitting fish pathogen. *BMC Microbiol* **14**, 1–6. (doi:10.1186/1471-2180-14-67)

41. McKenzie, V. J. & Townsend, A. R. 2007 Parasitic and Infectious Disease Responses to Changing Global Nutrient Cycles. *EcoHealth* **4**, 384–396. (doi:10.1007/s10393-007-0131-3)

42. Johnson, P. T. J., Chase, J. M., Dosch, K. L., Hartson, R. B., Gross, J. A., Larson, D. J., Sutherland, D. R. & Carpenter, S. R. 2007 Aquatic eutrophication promotes pathogenic infection in amphibians. *P Natl Acad Sci* **104**, 15781–15786. (doi:10.1073/pnas.0707763104)

43. Ketola, T., Mikonranta, L., Laakso, J. & Mappes, J. 2016 Different food sources elicit fast changes to bacterial virulence. *Biol. Lett.* **12**, 20150660. (doi: 10.1016/j.epidem.2009.04.003)

43. Anttila, J., Ruokolainen, L., Kaitala, V. & Laakso, J. 2013 Loss of Competition in the Outside Host Environment Generates Outbreaks of Environmental Opportunist Pathogens. *PLoS ONE*

8, e71621. (doi:10.1371/journal.pone.0071621.s004)

45. Menneratt, A., Hamre, L., Ebert, D., Nilsen, F., Dávidová, M. & Skorping, A. 2012 Life history and virulence are linked in the ectoparasitic salmon louse Lepeophtheirus salmonis. *J Evol. Biol.* **25**, 856–861. (doi:10.1111/j.1420-9101.2012.02474.x)

46. Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A. & Laxminarayan, R. 2015 Global trends in antimicrobial use in food animals. *P.. Natl. Acad. Sci.*, 201503141. (doi:10.1073/pnas.1503141112)

47. Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W. I., Takasu, H., Wada, S., Suzuki, S. & Virta, M. 2011 Tetracycline Resistance Genes Persist at Aquaculture Farms in the Absence of Selection Pressure. *Environ. Sci. Technol.* **45**, 386–391. (doi:10.1021/es102725n)

48. Di Cesare, A., Luna, G. M., Vignaroli, C., Pasquaroli, S., Tota, S., Paroncini, P. & Biavasco, F. 2013 Aquaculture Can Promote the Presence and Spread of Antibiotic-Resistant Enterococci in Marine Sediments. *PLoS ONE* **8**, e62838. (doi:10.1371/journal.pone.0062838.t003)

49. Schubert, A. M., Sinani, H. & Schloss, P. D. 2015 Antibiotic-Induced Alterations of the Murine Gut Microbiota and Subsequent Effects on Colonization Resistance against Clostridium difficile. *MBio* **6**, e00974–15. (doi:10.1128/mBio.00974-15)

50. Smith, K. F., Goldberg, M., Rosenthal, S., Carlson, L., Chen, J., Chen, C. & Ramachandran, S. 2014 Global rise in human infectious disease outbreaks. *J. Royal Soc. Interface* **11**, 20140950–20140950. (doi:10.1016/j.arcmed.2005.06.005)

51. Altizer, S., dobson, A., Hosseini, P., Hudson, P., Pascual, M. & Rohani, P. 2006 Seasonality and the dynamics of infectious diseases. *Ecol. Lett.* **9**, 467–484. (doi:10.1111/j.1461-0248.2005.00879.x)

52. Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S. & Samuel, M. D. 2002 Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158–2162. (doi:10.1126/science.1063699)

53. Karvonen, A., Rintamäki, P., Jokela, J. & Valtonen, E. T. 2010 Increasing water temperature and disease risks in aquatic systems: climate change increases the risk of some, but not all, diseases. *Int. J. Parasitol.* **40**, 1483–1488. (doi:10.1016/j.ijpara.2010.04.015)

54. Gardner, A., West, S. A. & Buckling, A. 2004 Bacteriocins, spite and virulence. *Proc. Royal Soc. B.* **271**, 1529–1535. (doi:10.1098/rspb.2004.2756)

55. Hawlena, H., Bashey, F., Mendes Soares, H. & Lively, C. M. 2010 Spiteful Interactions in a Natural Population of the Bacterium Xenorhabdus bovienii. *Am. Nat.* **175**, 374–381. (doi:10.1086/650375)

56. Bashey, F., Young, S. K., Hawlena, H. & Lively, C. M. 2012 Spiteful interactions between sympatric natural isolates of Xenorhabdus bovienii benefit kin and reduce virulence. *J. Evol. Biol.* **25**, 431–437. (doi:10.1111/j.1420-9101.2011.02441.x)

57. Cordero, O. X., Wildschutte, H., Kirkup, B., Proehl, S., Ngo, L., Hussain, F., Le Roux, F., Mincer, T. & Polz, M. F. 2012 Ecological populations of bacteria act as socially cohesive

600 units of antibiotic production and resistance. *Science* **337**, 1228–1231.
601 (doi:10.1126/science.1219385)

602 58. Pérez-Gutiérrez, R.-A. et al. 2012 Antagonism influences assembly of a Bacillus guild in a
603 local community and is depicted as a food-chain network. *The ISME Journal* **7**, 487–497.
604 (doi:10.1038/ismej.2012.119)

605 59. Staves, P. A. & Knell, R. J. 2010 VIRULENCE AND COMPETITIVENESS: TESTING THE
606 RELATIONSHIP DURING INTER- AND INTRASPECIFIC MIXED INFECTIONS
607 *Evolution* **64-9**:2643-2652

608 60. Cornforth, D. M. & Foster, K. R. 2013 Competition sensing: the social side of bacterial stress
609 responses. *Nat Rev Micro* **11**, 285–293. (doi:10.1038/nrmicro2977)

610 61. Buckling, A. & Brockhurst, M. A. 2008 Kin selection and the evolution of virulence. *Heredity*
611 **100**, 484–488. (doi:10.1038/sj.hdy.6801093)

612 62. Anacker, R. L. & Ordal, E. J. 1959 Studies on the Myxobacterium Chondrococcus
613 columnaris: II. Bacteriocins. *J. Bacteriol.***78**, 33–40.

614 63. Garbutt, J., Bonsall, M. B., Wright, D. J. & Raymond, B. 2011 Antagonistic competition
615 moderates virulence in Bacillus thuringiensis. *Ecol. Lett.* **14**, 765–772.

616 64. Pallen, M. J. & Wren, B. W. 2007 Bacterial pathogenomics. *Nature* **449**, 835–842.
617 (doi:10.1038/nature06248)

618 65. Staroscik, A. M., Hunnicutt, D. W., Archibald, K. E. & Nelson, D. R. 2008 Development of
619 methods for the genetic manipulation of Flavobacterium columnare. *BMC Microbiol* **8**, 115.
620 (doi:10.1186/1471-2180-8-115)

621 66. Li, N., Qin, T., Zhang, X. L., Huang, B., Liu, Z. X., Xie, H. X., Zhang, J., McBride, M. J. &
622 Nie, P. 2015 Development and use of a gene deletion strategy to examine the two chondroitin
623 lyases in virulence of Flavobacterium columnare. *Appl. Environ. Microbiol.*
624 (doi:10.1128/AEM.01586-15)

625 67. Tekedar, H. C. et al. 2012 Genome Sequence of the Fish Pathogen Flavobacterium columnare
626 ATCC 49512. *J.Bacteriol.* **194**, 2763–2764. (doi:10.1128/JB.00281-12)

627 68. Robertsen, B. 2011 Can we get the upper hand on viral diseases in aquaculture of Atlantic
628 salmon? *Aquaculture Res.* **42**, 125–131. (doi:10.1111/j.1365-2109.2010.02671.x)

629 69. Leung, T. L. F. & Bates, A. E. 2012 More rapid and severe disease outbreaks for aquaculture
630 at the tropics: implications for food security. *J. Appl. Ecol.* **50**, 215–222. (doi:10.1111/1365-
631 2644.12017)

632 70. FAO 2014 The state of world fisheries and aquaculture, opportunities and challenges. Food
633 and Agriculture Organization of the United Nations, Rome, Italy.

634

635 **TABLES**

636 **Table 1.** *Flavobacterium columnare* strains isolated in 2003-2010 and used in this study. Genetic
637 group is based on MLSA analysis which corresponds with the ARISA genotyping used with strains
638 in Table 2 [31].

Isolation source	Bacterial strain	Year of isolation	Fish species/water sample	Genetic group
A, fish farm Central Finland	B431	2003	Grayling *Thymallu thymallus*	A
	B067	2007	Trout *Salmo trutta*	A
	B185	2008	Rearing tank water	G
B, fish farm Central Finland	H2	2003	Rainbow trout *Oncorhynchus mykiss*	H
	B429	2003	Pikeperch *Zander lucioperca*	H
	B430	2003	Pikeperch *Z. lucioperca*	E
	B425	2007	Rainbow trout *O. mykiss*	
	B245	2009	Rearing tank water	C
	B259	2009	Rearing tank water	C
	B402	2010	Whitefish *Coregonus lavaretus*	C
	B366	2010	Outlet water of a fish farm	C
C, Nature (Lake)	B405	2010	Lake Jyväsjärvi	C
D, Nature (River)	B407	2010	River upstream the fish farm B	G
E, fish farm Northern Finland	B428	2006	Atlantic salmon *Salmo salar*	
	B426	2006	Atlantic salmon *S. salar*	C
	B420	2009	Atlantic salmon *S. salar*	G
	B421	2009	Atlantic salmon *S. salar*	C

639
640

641 **Table 2. *Flavobacterium columnare* strains used in this study.** The strains were isolated from
642 nature, from river upstream of a fish farm (inlet water) in Central Finland (farm B in Table 1) and
643 from the outlet water or from the river immediately downstream of the outlet water tube of the same
644 farm of the farm in summer 2010. Genetic grouping is based on Automated Ribosomal Intergenic
645 Spacer Analysis (ARISA).

Isolate	Inlet water	Outlet water	Genetic group	Time of isolation (2010)	Reference
B395	X[a]		G	21st June	[29]
B396	X[a]		A	"	"
B397	X[b]		C	"	"
B398	X[b]		A	"	"
B400	X[b]		A	19th July	"
B404	X[a]		C	2nd August	"
B355	X		A	"	This study
B406	X[b]		C	16th August	[29]
B407	X[b*]		G	"	"
B339		X[c]	C	5th July	This study
B340		X[c]	C	"	"
B350		X[a]	E	2nd August	"
B351		X[a]	E	"	"
B366		X[c]	C	"	"
B370		X[b]	E	16th August	"
B374		X[b]	E	"	"
B375		X[b]	E	"	"
B379		X[c]	E	30th August	"

[a] river, biofilm

[b] river, free water

[*] 400m upstream of the water intake

[c] outlet water of the farm

646

647 **Table 3.** Effects of the average year of isolation and the difference of the isolation years on growth
648 inhibition (i.e. interference, studied on agar plates) and competition (interaction studied in liquid
649 medium) in pairs of *Flavobacterium columnare* isolates, collected during disease epidemics in 2003-
650 2010.
651

Inhibition

Parameter	*Estimate*	*Std. Error*	*t*	*Sig.*
Average year of isolation	0.0034	0.002	2.078	0.039
Difference in years of isolation	-0.0001	0.002	0.764	0.446

Interaction index

Parameter	*Estimate*	*Std. Error*	*df*	*t*	*Sig.*
Average year of isolation	-0.0255	0.009	104.707	-2.759	0.007
Difference in years of isolation	0.0024	0.007	103.641	0.355	0.724
(Strain combination 0.0215 s.e:0.0036, Wald Z:5.911, p<0.001, Residual: 0.008, s.e.:0.001, Wald Z:6.959, p<0.001)					

652
653
654
655
656
657
658
659
660
661
662
663
664
665

666 **Table 4.** Pairwise comparisons of risk of inhibition (interference competition) by inlet water and
667 outlet water strains in different combinations of donors and receivers (each reciprocal combination
668 considered as separate combination). I = inlet water strains isolated from upstream of a fish farm, O
669 = strains isolated from outlet water or downstream of the farm.
670

	Donor O, Reciever I	Donor I, Reciever O	Donor O, Reciever O
Donor I, Reciever I	z=1.084, p=0.2790	z=0.098, p=0.922	z=-1.086 , p=0.278
Donor O, Reciever I		z=-0.986 p=0.325	**z=-2.154, p=0.033**
Donor I, Reciever O			z= 1.183, p=0.238

671
672

673 **Table 5.** Results of the mixed model analysis exploring the growth differences between the inlet
674 water and the outlet water isolates (Location) of *F. columnare* measured in high and low resource
675 concentrations. The model also contains a random effect of the strain identity to control for the non-
676 independency of observations arising from repeated growth measurements.
677

	Maximal growth rate (change in OD h -1)		
	F	df1,df2	p
Location	0.968	1,16.054	0.34
Resource concentration	27.113	1,163.047	<0.001
Location x Resource concentration	5.826	1,163.047	0.017
Strain identity	Wald Z:	2.477	0.013

	Yield		
	F	df1,df2	p
Location	6.372	1,15.989	0.023
Resource concentration	173.173	1,163.002	<0.001
Location x Resource concentration	0.004	1,163.002	0.947
Strain identity	Wald Z:	2.729	0.006

	AUC		
	F	df1,df2	p
Location	7.724	1,15.989	0.013
Resource concentration	136.565	1,163.002	<0.001
Location x Resource concentration	1.302	1,163.002	0.255
Strain identity	Wald Z:	2.722	0.006

678
679

680 **FIGURE LEGENDS**

681

682 **Figure 1.** Virulence of *Flavobacterium columnare* in experimentally infected zebra fish (*Danio*
683 *rerio*) **A)** The mean longevity (±S.E., open squares, left axis) and survival percentage (dark circles,
684 right axis) of the infected zebra fish (n=10 in each bacterial exposure) after exposure to bacterial
685 strains isolated in 2003-2010. **B)** Survival percentage of zebra fish (n=14 in each bacterial exposure)
686 after exposure to bacterial strains isolated from inlet water (B355-B407) and outlet water (B399-
687 B397) of a fish farm (dark circles for individual strains, open circles for mean survival±S.E.).

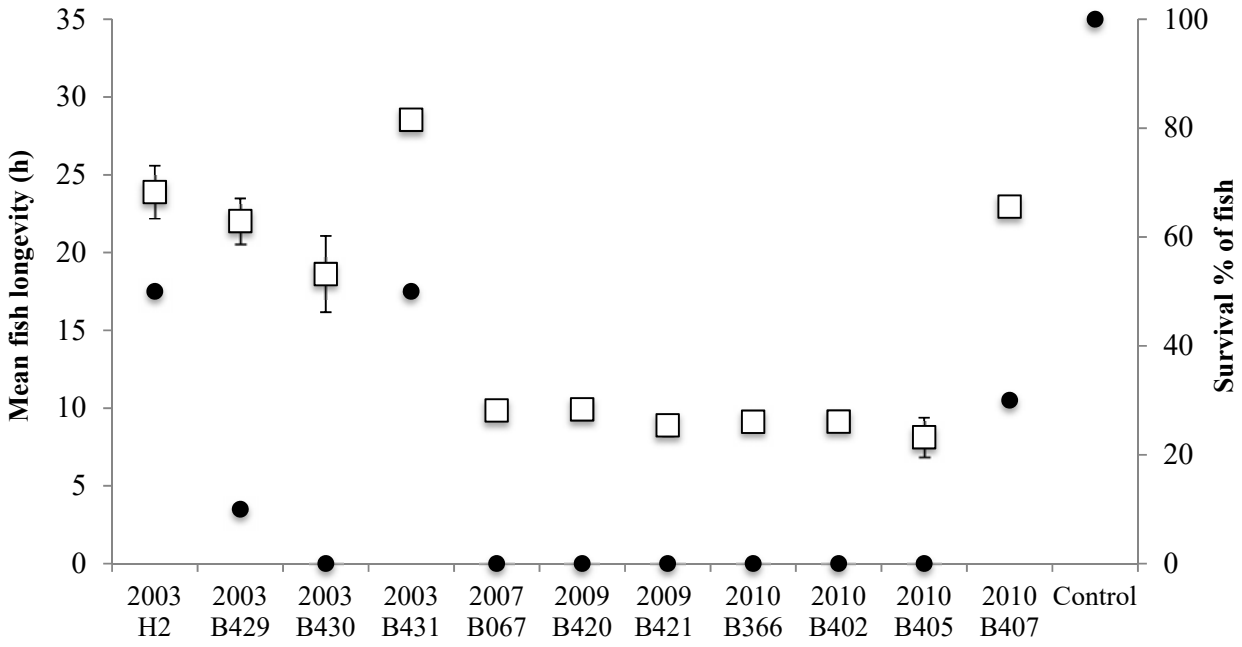
688

689 **Figure 2.** Changes in (arcsin-transformed) interaction index of the competing pairs of
690 *Flavobacterium columnare* strains on limited resources in relation to the mean isolation time of the
691 pair of isolates. Lower index indicates higher competition. Circle indicates the mean of the two
692 replicates of the tested *F. columnare* strain pairs.

693

FIGURE 1

A)


Mean fish longevity (h)
Survival % of fish
35
30
25
20
15
10
5
0
100
80
60
40
20
0
2003 H2
2003 B429
2003 B430
2003 B431
2007 B067
2009 B420
2009 B421
2010 B366
2010 B402
2010 B405
2010 B407
Control

B)

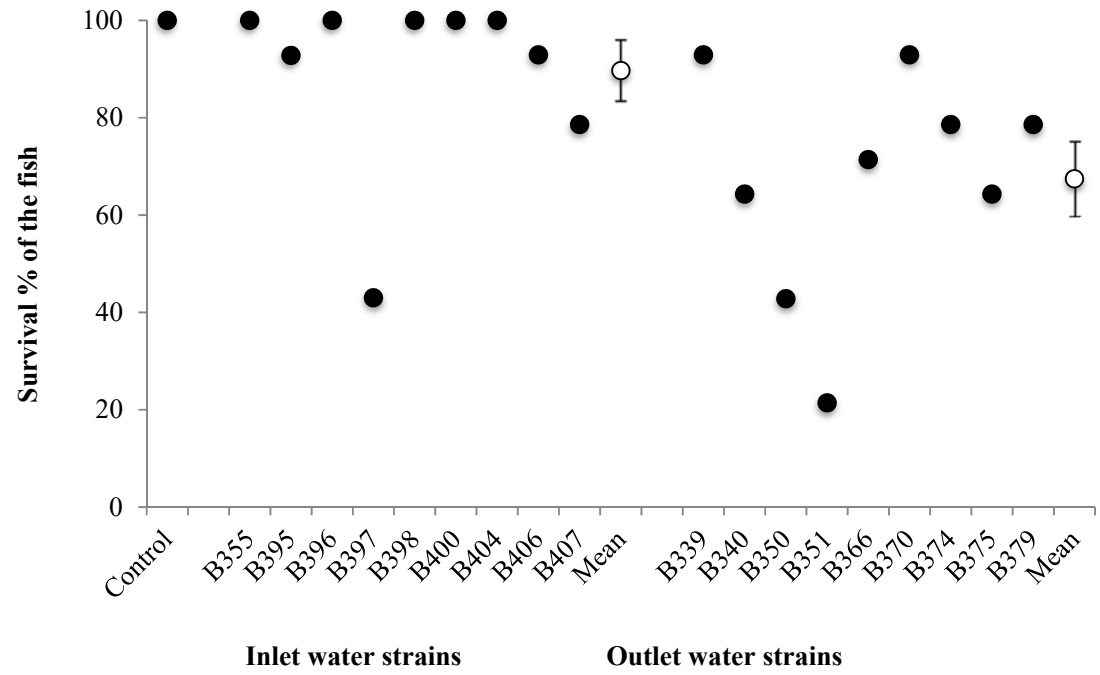
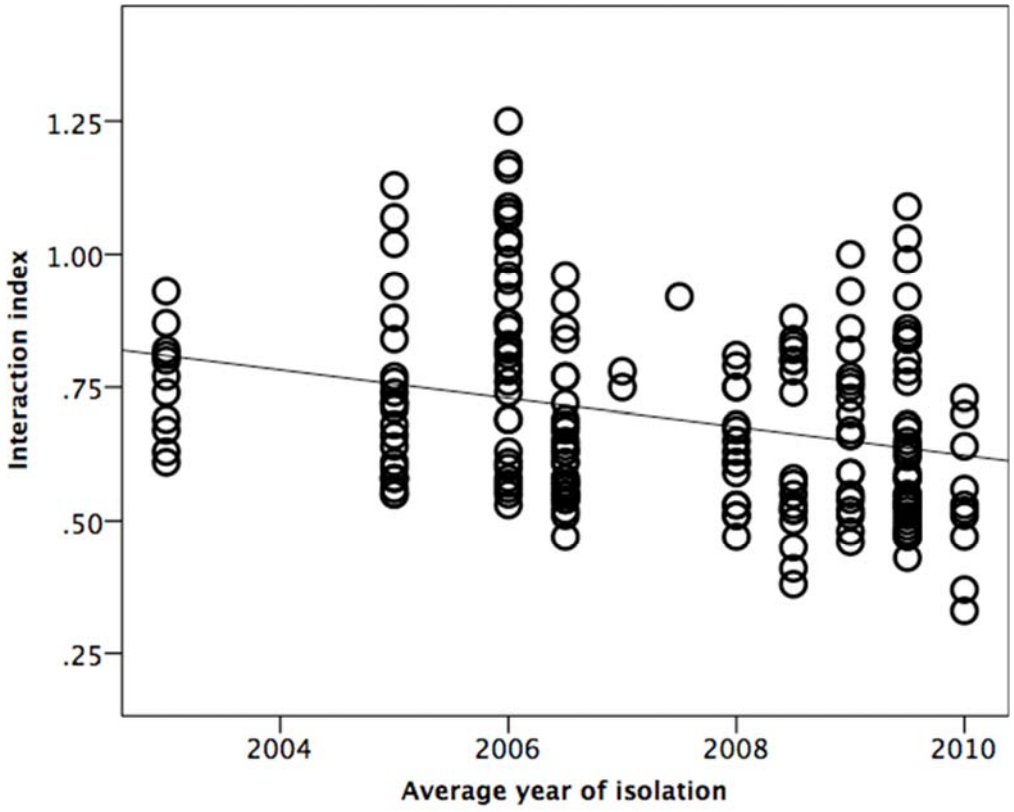

Survival % of the fish
100
80
60
40
20
0
Control
B355
B395
B396
B397
B398
B400
B404
B406
B407
Mean
B339
B340
B350
B351
B366
B370
B374
B375
B379
Mean
Inlet water strains
Outlet water strains

FIGURE 2


1.25
1.00
.75
.50
.25
Interaction index
2004
2006
2008
2010
Average year of isolation

709

710 **Supplementary material**

711

712 Intensive aquaculture selects for increased virulence and interference competition in bacteria

713

714 Lotta-Riina Sundberg*, Tarmo Ketola, Elina Laanto, Hanna Kinnula, Jaana K.H. Bamford, Reetta
715 Penttinen, and Johanna Mappes

716

Supplementary methods

Genetic characterization of bacteria

The strains isolated in 2003-2010 were genetically characterized in a previous study using the Multilocus Sequence Analysis developed for *F. columnare* [1]. The inlet water strains were characterized in an earlier study [2] with the Automated Ribosomal Intergenic Spacer Analysis (ARISA), which corresponds with the MLSA typing [1]. The outlet water *F. columnare* strains and one inlet water strain (B355) were genotyped in this study, essentially as described earlier [2,3]. Shortly, DNA from overnight cultures was isolated with Fermentas Life Sciences GeneJET Genomic DNA Purification Kit (#K0722) according to manufacturer's instructions. Ribosomal intergenic spacer region was amplified by PCR using primers rD1f (5'-GGCTGGATCACCTCCTT-3', with 6FAM label, [4] and 23Sr (5'-GGGTTBCCCCATTCRG-3', [5]. The PCR products were denatured with formamide mixed with GeneScan^{TM} 1200 LIZ Size Standard (1:20 mixture), separated with an ABI Prism 3130xl Genetic Analyser, and visualized with GeneMapper v.5.0 software (all Applied Biosystems, Carlsbad, California, USA).

732

733

734 **Supplementary Table 1.** *Flavobacterium columnare* occurrence (indicated by grey bar) at Fish farm
735 B on sampling dates in 2010. The asterisk indicates that an obtained bacterial isolate was used in the
736 experiments (details in Table 2 in main text).

737

Date	Inlet water	Outlet water	Water temperature°C
February 23 th			0.5
April 26 th			
May24 th			
June 8 th			14
June 21 st	*		15.5
July 5 th		*	20.5
July 12 th			
July 19 th	*		24.8
July 26 th			22.2
August 2 nd	*	*	22
August 16 th	*	*	20.8
August 30 th		*	15.8
October 11 th			8
November 15 th			1.7

738

739

Supplementary Table 2. The inhibition profiles of *Flavobacterium columnare* strains isolated **a)** in 2003-2010 and **b)** from inlet water (B355-B407) and outlet water (B339-B379) of a fish farm during summer 2010. The number in the cell represents the frequency of inhibition. Experiments were done in three (strains 20013-2010) or four (inlet, outlet waters strains) independent replicates. Supernatant of each overnight grown bacteria ('donors', top row) were spotted on each bacteria mixed in soft agar ('recipients', columns below) in a double layer plate assay. The presence of inhibition was recorded after 48h.

a)

			2003 A	2003 B	2003 B	2003 B	2006 E	2006 E	2007 B	2007 A	2009 E	2009 E	2009 A	2009 B	2009 B	2010 C	2010 D	2010 B	2010 B
Year	**Farm**	**Code**	B431	H2	B429	B430	B428	B426	B425	B067	B420	B421	B185	B245	B259	B405	B407	B402	B366
2003	A	B431		0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	2
2003	B	H2	0		0	3	0	0	1	0	0	0	0	1	0	2	0	1	2
2003	B	B429	0	0		2	0	0	0	0	0	0	0	0	0	2	0	1	0
2003	B	B430	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0
2006	E	B428	0	0	0	0		0	0	0	0	0	2	0	0	0	1	0	0
2006	E	B426	0	0	0	0	0		0	0	0	0	1	0	0	0	0	0	0
2007	B	B425	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0
2007	A	B067	0	0	0	2	0	1	1		0	0	1	0	0	2	0	0	1
2009	E	B420	0	0	0	1	0	0	2	0		0	0	1	0	1	0	0	3
2009	E	B421	0	0	0	1	0	0	0	0	1		1	0	0	1	0	0	0
2009	A	B185	0	0	1	1	1	0	1	0	0	0		1	1	1	0	1	2
2009	B	B245	0	0	0	0	0	0	0	0	1	0	1		0	0	0	0	0
2009	B	B259	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0
2010	C	B405	0	0	0	0	0	0	0	0	1	0	0	0	0		0	0	0
2010	D	B407	1	0	1	3	1	1	3	0	0	0	0	2	3	1		2	2
2010	B	B402	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0		1
2010	B	B366	0	0	0	0	0	0	0	0	2	0	1	0	0	0	1	0	

b)

		B355	B395	B396	B397	B398	B400	B404	B406	B407	B339	B340	B350	B351	B366	B370	B374	B375	B379
Inlet	B355		0	0	2	1	0	0	1	0	1	2	0	1	0	1	2	0	1
Inlet	B395	4		3	2	2	3	3	4	4	2	4	0	3	3	3	4	1	4
Inlet	B396	1	0	1	1	1	0	1	2	2	1	2	0	2	1	2	1	1	1
Inlet	B397	1	2	0		0	0	0	1	2	0	1	0	1	0	0	2	0	1
Inlet	B398	2	1	0	1		1	1	2	1	0	2	0	1	3	2	2	1	1
Inlet	B400	2	1	0	1	0		1	1	1	0	2	0	2	2	1	1	1	1
Inlet	B404	0	1	0	0	0	0		3	1	0	0	0	1	0	1	1	0	1
Inlet	B406	3	1	1	0	1	0	0		1	1	0	0	1	0	1	3	0	1
Inlet	B407	1	2	0	1	0	0	1	2		0	0	0	0	0	1	1	0	1
Outlet	B339	1	1	0	0	0	0	0	0	1		2	0	1	0	2	1	0	1
Outlet	B340	0	1	0	0	0	0	0	1	0	0		0	1	0	1	1	0	0
Outlet	B350	1	1	0	1	1	0	1	2	0	1	2		1	0	1	3	0	1
Outlet	B351	1	0	0	3	0	0	2	2	0	1	3	0		2	1	3	0	0
Outlet	B366	2	4	2	1	2	1	1	2	2	2	2	0	1		3	2	0	1
Outlet	B370	0	1	1	1	0	0	2	3	0	1	3	0	0	0		0	0	0
Outlet	B374	0	0	0	2	0	0	2	3	0	0	1	0	0	0	0		0	0
Outlet	B375	1	1	0	3	1	0	1	3	0	0	1	0	0	1	1	1		0
Outlet	B379	0	1	0	2	1	0	1	2	0	0	2	0	0	1	2	1	0	

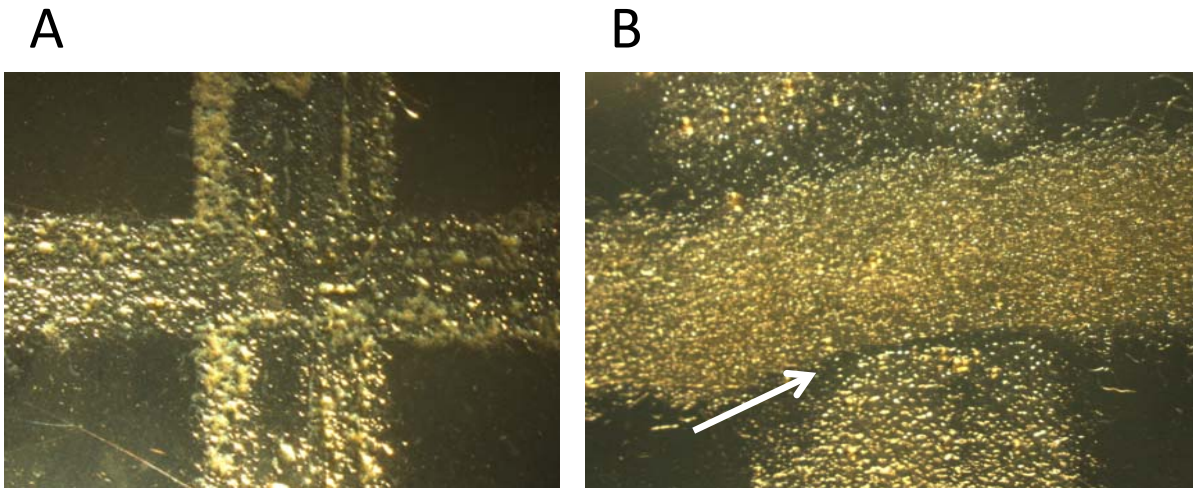
Supplementary Table 3. Effects of the year of isolation and the strain identity on growth of *Flavobacterium columnare* strains isolated in 2003-2010.

Maximal growth rate (change in OD h -1)	*Estimate*	*Std. Error*		*Sig.*
Year of isolation	-0.007	0.007	F:-0.977	0.346
Strain ID	0.006	0.002	Wald Z: 2.504	0.012

Yield	*Estimate*	*Std. Error*		*Sig.*
Year of isolation	-0.007	0.007	F:-0.977	0.346
Strain ID	0.006	0.002	Wald Z: 2.502	0.012

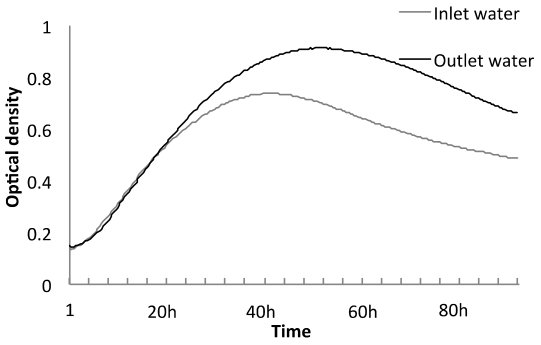
Area under curve	*Estimate*	*Std. Error*		*Sig.*
Year of isolation	-12.93	22.99	F:-0.562	0.584
Strain ID	62088.88	24388.15	Wald Z: 2.546	0.011

Supplementary Figure 1. Growth inhibition similar to Dienes lines (indicated by white arrow) in the intersection of agar plate culture of *Flavobacterium columnare* strains. A) Control B185 + B185, B) B185 (horizontal) and B067 (vertical).

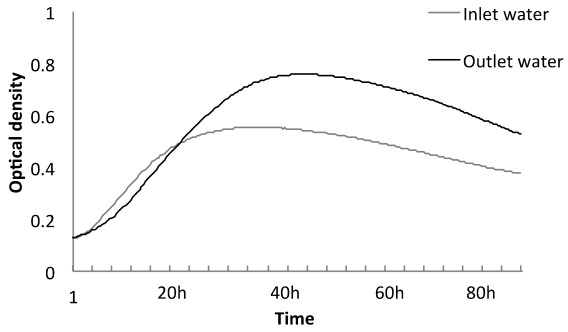


Supplementary Figure 2. Growth (measured as optical density over time) of the inlet water and the outlet water *Flavobacterium columnare* strains in a) standard culture medium and b) 0.5x diluted medium.

a)

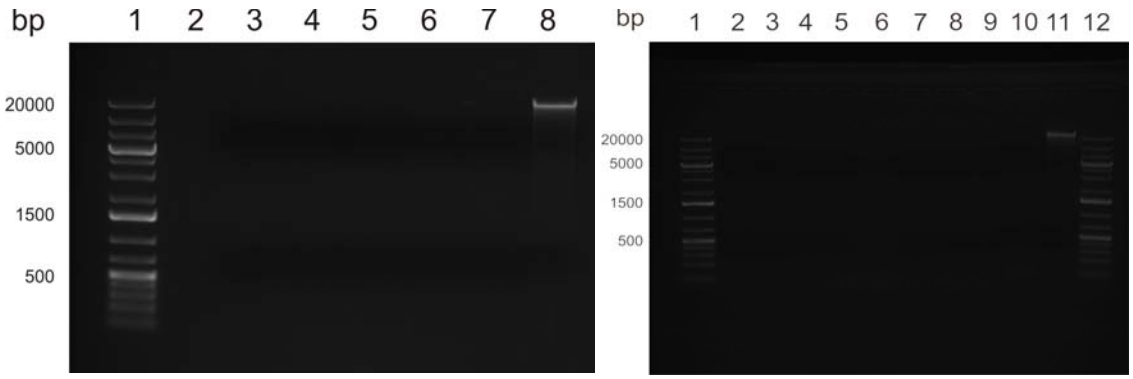


b)



Supplementary Figure 3. Plasmid screening for *F. columnare* isolates. Lane 1: GeneRuler™ 1kb Plus DNA Ladder (Fermentas). A) Lanes 2 to 7: B067, B185, B245, B405, B407, B420. Lane 8: *Flavobacterium* sp. B330 (positive control). B) Lanes 1 and 12: GeneRuler™ 1kb Plus DNA Ladder (Fermentas). Lanes 2 to 10: B366, B404, B375, B349, B406, B339, B400, B407, B395. Lane 11: B330 *Flavobacterium* sp. B330 (positive control).

A **B**



SUPPLEMENTARY REFERENCES

1. Ashrafi, R., Pulkkinen, K., Sundberg, L.-R., Pekkala, N. & Ketola, T. In press. A multilocus sequence analysis scheme for characterization of Flavobacterium columnare isolates. *BMC Microbiol*

2. Kunttu, H. M. T., Sundberg, L.-R., Pulkkinen, K. & Valtonen, E. T. 2012 Environment may be the source of Flavobacterium columnare outbreaks at fish farms. *Environmental Microbiology Reports* **4**, 398–402. (doi:10.1111/j.1758-2229.2012.00342.x)

3. Suomalainen, L. R., Kunttu, H., Valtonen, E. T., Hirvela-Koski, V. & Tiirola, M. 2006 Molecular diversity and growth features of Flavobacterium columnare strains isolated in Finland. *Dis Aquat Organ* **70**, 55–61.

4. Borneman, J. & Triplett, E. W. 1997 Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* **63**, 2647–2653.

5. Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. 1991 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697–703.