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Year: 2016

Version:

Please cite the original version:

Penttinen, R., Kinnula, H., Lipponen, A., Bamford, J., & Sundberg, L.-R. (2016). High Nutrient Concentration Can Induce Virulence Factor Expression and Cause Higher Virulence in an Environmentally Transmitted Pathogen. Microbial Ecology, 72(4), 955-964. https://doi.org/10.1007/s00248-016-0781-1

High nutrient concentration can induce virulence factor expression and cause higher virulence in an environmentally transmitted pathogen

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ABSTRACT

Environmentally transmitted opportunistic pathogens shuttle between two substantially different environments: outside-host and within-host habitats. These environments differ from each other especially with respect to nutrient availability. Consequently, the pathogens are required to regulate their behavior in response to environmental cues in order to survive, but how nutrients control the virulence in opportunistic pathogens is still poorly understood. In this study we examined how nutrient level in the outside-host environment affects the gene expression of putative virulence factors of the opportunistic fish pathogen *Flavobacterium columnare*. The impact of environmental nutrient concentration on bacterial virulence was explored by cultivating the bacteria in various nutrient conditions, measuring the gene expression of putative virulence factors with RT-qPCR, and finally, experimentally challenging rainbow trout (*Oncorhynchus mykiss*) fry with these bacteria. Our results show that increased environmental nutrient concentration can increase the expression of putative virulence genes, chondroitinase (*cslA*) and collagenase, in the outside-host environment and may lead to more rapid fish mortality. These findings address that the environmental nutrients may act as significant triggers of virulence gene expression and therefore contribute to the interaction between an environmentally transmitted opportunistic pathogen and its host.

INTRODUCTION

Environmentally transmitted opportunistic pathogens can often survive and replicate in the environment outside the host [5]. Thus, the nutrients of the surrounding environment may play a fundamental role in pathogen survival and significantly influence disease epidemics. High nutrient levels have been shown to be linked with increased virulence, possibly via an increased pathogen growth rate or decreased oxygen levels stressing the host [6, 33, 58]. Also, nutrient quality can have large-scale effects on host-pathogen dynamics in nature [17]. In environmentally transmitted pathogens, the transitions between the outside-host environment and the host expose the pathogens to various

conditions, which may influence the emergence of disease outbreaks. Bacteria respond to environmental cues, such as elevated nutrient sources, and change their gene expression accordingly. The driving force behind bacterial pathogenicity is acquisition of host-derived nutrients for bacterial growth and survival. To achieve this, pathogens use virulence factors to colonize the host, avoid the host immune response and, finally, access the host tissues for nutrients [8]. Many bacterial pathogens secrete proteases that often have a role in bacterial pathogenesis [29]. These tissue-degrading enzymes provide nutrients for bacterial growth during infection and transmission ([30]; see also [43] for a review). So far, we have a limited understanding of how environmental nutrient levels affect the expression of bacterial virulence factors, i.e. enzymes for host-tissue degradation, which may not be essential outside the host. Hence, understanding how the environment influences the virulence factor production and its regulation can provide tools for disease management and also deepen our knowledge of host-pathogen interactions.

Flavobacterium columnare (Bacteroidetes) is a member of the natural aquatic microbial community [22, 42], but it is also an important fish pathogen worldwide, causing significant economic losses for the freshwater aquaculture industry [11, 40, 56]. *F. columnare* has been shown to persist long periods in the environment outside the host [2, 49] and it has been suggested that environment can serve as a reservoir of the bacteria between the outbreak seasons [22, 36]. Despite its importance, the virulence mechanisms and their expression of *F. columnare* are still largely unknown. Especially in the case of columnaris disease, in which the symptoms can be seen as skin lesions, fin erosion and gill necrosis [3, 54], enzymes targeting host tissue degradation may play a considerable role. Proteomics and suppression subtractive hybridization (SSH) [14, 32, 35] have revealed some potential factors involved with *F. columnare* virulence, such as the tissue-degrading enzymes chondroitinase (chondroitin AC lyase) and collagenase [35]. These enzymes degrade chondroitin sulfate and collagen, respectively, which are both widely found in animal connective tissues. Chondroitinase production has been connected to the virulence of *F. columnare* [23, 48, 51], and collagenolytic activity has been shown to be involved with bacterial virulence in many other pathogenic bacteria [18]. Furthermore, when grown under laboratory conditions, *F. columnare* can display three colony morphotypes: Rhizoid (Rz) and its derivatives Rough (R) and Soft (S). Of these colony types, only the ancestral Rz type is highly virulent [24, 27, 28]. Comparison of virulent and nonvirulent colony types in transcriptional level can help to identify genetic factors that are needed to produce bacterial infection.

In this study, we developed an RT-qPCR protocol with two reference genes for studying the gene expression in *F. columnare.* Reverse transcription quantitative PCR (RT-qPCR) assays can be used to quantitatively measure the expression of specific genes of interest, such as those encoding proteases or other virulence factors. Primer design and optimization, high-quality template material and normalization with more than one stably expressed reference gene are all crucial elements in RT-qPCR to generate accurate gene expression results [7]. The expression of the tissue-degrading enzymes chondroitinase and collagenase were determined in virulent (Rz) and nonvirulent (R and S) *F. columnare* B067 morphotypes [27] in liquid cultures and on agar plates, in which the nutrient concentration of the plates was manipulated. Furthermore, we analyzed whether the nutrient concentration of the culture medium has a direct effect on bacterial virulence using two *F. columnare* strains in an experimental infection of rainbow trout fry. The aim of this study was to identify

the outside-host conditions favoring the expression of tissue-degrading enzymes in order to understand factors that increase the virulence of this opportunistic pathogen.

RESULTS

Validation of reference genes for qPCR assay

The stability of five reference gene candidates (*16S rRNA,* 16S ribosomal RNA; *gapdh*, glyceraldehyde 3-phosphate dehydrogenase*; glyA,* serine hydroxymethyltransferase*; rplQ,* ribosomal protein L17*; rpoD,* RNA polymerase sigma factor) was evaluated in a sample set (N=27) in GenEx v6.0 (MultiD Analyses) with the integrated geNorm and NormFinder methods [1, 55]. According to both methods, the best expression stability was obtained for the *gapdh* and *glyA* genes. M value of 0.52 for both genes was calculated with geNorm and standard deviations 0.19 and 0.26 for *glyA* and *gapdh* with Normfinder, respectively; Fig. S1. The stability was then calculated with geNorm method for the entire dataset, including the samples of Rz, R and S types grown in 1x liquid Shieh broth and on 0.5x, 1x and 2x Shieh agar, giving the M value of 0.69 for *gapdh* and *glyA* which were selected as reference genes and used for normalization of the gene expression data (Table S1).

Expression of chondroitinase and collagenase in liquid culture

F. columnare morphotypes Rz, R and S were grown in Shieh broth and their gene expressions during the logarithmic growth phase were compared. The expression of chondroitinase (encoded by gene *cslA*) differed significantly between the *F. columnare* morphotypes ($F = 21.189$, $df = 2$, $p < 0.001$), being highest in the Rz morphotype as compared to the R and S types (LSD post hoc test, $p=0.002$ and $p<0.001$, respectively). Collagenase expression did not differ between the morphotypes (Fig. 1).

The DNA sequences of chondroitinase and collagenase were analyzed in all *F. columnare* B067 colony types (Rz, R and S) to search for genetic differences. Chondroitinase and collagenase genes and their predicted promoter regions were 100 % identical in all three colony types. Sequences are submitted in GenBank under accession numbers KR014145 for chondroitinase and KR014146 for collagenase.

Expression of chondroitinase and collagenase on agar plates with various nutrient levels

F. columnare morphotypes Rz, R and S were grown on Shieh agar plates with various Shieh concentrations (0.5x, 1x or 2x). After 2 days of incubation, the expressions of chondroitinase and collagenase in different colony types were compared. Colony type had a significant impact on the expression of chondroitinase (F 5.094, $p = 0.010$), the expression being highest in the virulent Rz type (Fig. 2; Table 1). In contrast to the liquid culture, on agar plates the collagenase expression differed between the colony types (F 7.283, $p = 0.002$), and the highest level of expression was observed in the nonvirulent R type. Interactions between the colony type and nutrients were not found (Table 1). Post hoc-comparisons revealed that a high nutrient concentration significantly increased the expression of chondroitinase in all morphotypes (Table 2). Similarly, a significant increase in collagenase expression was observed in the virulent Rz morphotype as a response to increased nutrient level, but not in the nonvirulent R. Gene expression patterns of nonvirulent S type in various conditions were similar to that of

Rz, however nutrients did not have statistically significant impact on gene expression in S type (Table 2).

Environmental nutrient level can increase bacterial virulence in experimental infection of rainbow trout

The virulence of two *F. columnare* strains (B067 and B185), both Rz type, that were cultured in various nutrient conditions: 2xN, 1x or 0.5xN Shieh (N referring to the modified concentration of peptone and yeast extract, see Supplementary Table S3) was studied by infection of rainbow trout fry. The mortality of the infected fish in the 26-hour experiment was 93.75-100 %. Bacteria that were cultured in high nutrient concentration (2xN Shieh) caused significantly faster fish mortality and more rapid onset of disease in both bacterial strains (Table 3, Fig. 3). Fish mortality in the control group, that had been challenged with growth medium only, reached ~45 %. However, the skin cultivations on Shieh agar plates from the infected fish were 98 % positive for *F. columnare*, while the bacterium was not detected in the cultivations from the control fish, indicating that the cause of death in the control group was most probably the stressful experimental conditions.

DISCUSSION

Opportunistic pathogens often have the ability to persist and transmit via the environment [5] that can substantially differ from the nutrient-rich within-host milieu. As the environment outside the host is unpredictable, the bacteria have to adapt rapidly to the surrounding conditions, e.g. temperature [16] or nutrients [21]. Furthermore, environmentally transmitted pathogens need to have capacity for the invasion when a potential host is encountered, but the production of virulence factors in the absence of a host is costly. Therefore, the expression of virulence factors has to be carefully regulated. Environmental signals can serve as triggers in switching the metabolic routes on and off, but the molecular-level knowledge of how nutrients in the outside-host environment affect the virulence mechanisms in environmentally transmitting pathogens is so far limited.

We developed an RT-qPCR method with two reference genes for studying the gene expression of the fish pathogen *F. columnare.* The expression of tissue-degrading enzymes chondroitinase and collagenase, that are likely involved in virulence [18, 23, 48, 51], was explored in the virulent Rz and nonvirulent R and S colony variants of *F. columnare* strain B067 (8-10). Furthermore, we examined how the nutrient conditions influence the expression of these enzymes in order to find associations with colony type and virulence. We found that the virulent Rz type bacteria expressed chondroitinase (*cslA)* significantly more as compared to the nonvirulent types, both in liquid and on agar plates. Surprisingly, the increased collagenase expression was not associated with the virulent Rz colony morphotype, but was observed in the nonvirulent R colony type. A significant increase in the expression of both tissue-degrading enzymes in response to increased nutrient concentration was detected, indicating that the gene expression may be regulated by the conditions of the outside-host environment. Finally, we found that the bacteria cultured in the high-nutrient medium were significantly more virulent in the natural fish host than those cultured in the low-nutrient medium.

Extracellularly secreted proteins and proteinases have a significant role in bacterial virulence because they act in the interface between the pathogen and the host organism [9, 30, 41]. Pathogenic species of phylum Bacteroidetes, such as *Porphyromonas gingivalis, Bacteroides fragilis* and *B. thetaiotamicron,* produce a variety of hydrolytic enzymes that degrade components of extracellular matrix of the host tissues [44, 53, 59]. Hence, in addition to acquisition of host-derived nutrients, these enzymes are important in host invasion and colonization [53]. The external symptoms caused by *F. columnare* infection, such as tissue lesions and erosion [40] reflect the importance of proteases for columnaris disease pathology. Indeed, proteolytic activities, including ability to degrade gelatin, casein, hemoglobin, fibrinogen and elastin, have been reported in *F. columnare* [4]. However, thus far only chondroitinase, an enzyme degrading chondroitin sulfates, the proteoglycan components of vertebrate connective tissues, has been connected to virulence of *F. columnare* [23, 48, 51]. Chondroitinase activity of the colony types has been studied previously and it has been concluded that the Rz type exhibits the highest enzymatic activity of chondroitin AC lyase [23]. In accordance with these previous studies, our current study links the high chondroitinase (encoded by the gene *cslA*) expression with virulence. RT-qPCR revealed that the chondroitinase expression was significantly higher in the virulent colony type Rz as compared to the nonvirulent types. It was reported recently that *F. columnare* produces also a second chondroitin lyase gene, *cslB* [31]. However, a deletion of *cslA* resulted in significant reduction in the lytic activity of chondroitinase, whereas *cslB* possessed only a minor role in the total chondroitinase activity [31].

Many pathogenic bacteria produce proteases with collagenolytic activity [18]. These enzymes degrade collagens, constituents of extracellular matrix (ECM) found in vertebrate tissues, and contribute significantly to host tissue damage [18]. For instance, Arg gingipains, that are virulence factors produced by a periodontal pathogen *P. gingivalis,* possess collagenolytic activity [19]. Collagenase has been suggested as a putative virulence factor in *F. columnare* based on its absence in *F. johnsoniae,* a nonvirulent member of Flavobacteria, as revealed by suppressive subtractive hybridization analysis [35]. However, the actual function and role of collagenase in *F. columnare* virulence have not been studied further. In the closely related fish pathogen, *Flavobacterium psychrophilum*, collagenase has been connected with virulence [34, 38] but opposite results have also been obtained [13, 50], and thus, its role in pathogenesis of *F. psychrophilum* has remained uncertain. Our results could not directly link collagenase with the virulent colony type of *F. columnare,* and it remains to be studied whether some other processes, such as temporal regulation of virulence factors (similar to e.g. *Staphylococcus aureus* virulence factors [37]) are associated with collagenase expression. Collagenase is an important virulence factor also in *Leptospira interrogans,* and interestingly, its expression is induced by contact with cell cultures [20]. Therefore our study cannot rule out the possible role of collagenase as a virulence factor in *F. columnare*, and its expression needs to be studied further in the presence of an appropriate substrate or host tissue. Furthermore, collagenolytic activity of *F. columnare* should be verified to find associations with virulence.

Bacteria sense properties of their physical and biological environment to take the best advantage of the available resources. Hence, local environmental cues have wide influences on bacterial physiology [46]. Environmental signals are recognized by systems that function in global coordination of gene regulation, e.g. carbon catabolite repression [15], nitrogen phosphotransferase system [39] and ppGpp [10] all of which are found in several bacterial species. With these multi-functional systems it has been

exemplified that virulence pathways are often integrated in the general metabolism of bacteria and exploit the signaling pathways crucial for metabolism of carbon and nitrogen, for example [10, 15, 39]. The presence of the host can be a signal for a pathogen to turn on the virulence factor expression. In *Bacteroides thetaiotaomicron*, the chondroitin lyase activity is induced by the presence of its substrate, chondroitin sulfate A [45], similarly as the collagenase expression is in *L. interrogans* mentioned above [20]. Given that the outside- and within-host habitats are different especially with respect to nutrient concentration, it is possible that nutrients contribute to the regulation of virulence gene expression. In general, the increase of nutrients is expected to influence disease dynamics via accelerating the pathogen growth rate and infective dose (26, 27). However, our study indicates a possibility, that high nutrient availability during outside-host growth could increase the virulence factor (*cslA*) expression, and directly prime bacterial virulence even in the absence of host. Changes in virulence were observed with bacteria grown under altered peptone and yeast extract concentrations, both of which are complex nutrient resources. Therefore, a more detailed exploration is needed to determine the specific components that contribute to the regulation of chondroitinase expression. Nevertheless, it has been shown that *F. columnare* can maintain high virulence also during starvation [49], but how a long-scale exposure to nutrient-limited conditions affect the virulence factor expression in *F. columnare* is not known.

The expression of chondroitinase and collagenase was measured using RT-qPCR, which can be used for mRNA quantification. However, the final enzymatic activity is dependent on the following steps, including the post-transcriptional and post-translational regulation and the accurate folding and modifications of the protein. Furthermore, the proteolytic enzymes that are needed for the host tissue destruction need to be successfully secreted to their final subcellular location, for which a functional secretion system is required. Therefore, the future studies may include biochemical quantification of the enzyme activity in different conditions to explore whether the high mRNA level reflects the true enzyme activity of the extracellular environment. In addition, a wider approach, such as transcriptome sequencing, could help to understand the wider role of environmental nutrients in the gene expression and regulation of *F. columnare*.

The pathogen-environment interaction is complex, and both the quantity and quality of the available nutrients can influence bacterial virulence [21]. In the current study, the increase in the general nutrient concentration significantly increased not only the expression of tissue-degrading enzymes, but also bacterial virulence. These findings may have significant impacts at the applied level, as the uneaten fish food, feces and carcasses offer nutrient resources for outside-host growth of *F. columnare* in the aquaculture rearing units [25, 57]. Furthermore, these conditions may preload the virulence machineries and thereby directly alter the disease dynamics.

MATERIALS AND METHODS

qPCR primer design and validation

Primers for amplifying and sequencing the gene of interest were designed using the whole genome sequence of *F. columnare* ATCC 49512 [52], as well as our own shotgun sequencing results. The primers were designed with Primer-BLAST

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of the primers used in this study are listed in Table S1. The specificities of the primer pairs were verified by inspecting the length of the PCR product in agarose gel electrophoresis and by melting curve analysis in CFX Manager™ Software v3.0 (for melt curves, see Fig. S2). Specific primer binding was also confirmed by sequencing the qPCR amplicon with an appropriate primer using Sanger method (see below). The resulting DNA sequences were imported into BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and correct binding was ensured if sequence originated from the relevant gene. The efficiency of each primer pair was determined by making a standard curve from a 1:5 diluted dilution series as a template and analyzed with CFX Manager™ Software v3.0 (Bio-Rad; Table S1).

The preliminary expression stability testing was first performed with five reference gene candidates (*16S rRNA; gapdh: glyA; rplQ; rpoD*) using a test sample set (N=27) that included samples from Rz, R and S colony types grown on 1x Shieh agar plates and in 1x Shieh liquid medium. The expression stabilities were measured via the geNorm [55] and NormFinder [1] methods, which are integrated in GenEx v6.0 (MultiD Analyses) (Fig. S1A and S1B). According to both methods, the *gapdh* and *glyA* genes possessed the best stabilities and were chosen to be used as reference genes in the expression analysis of the entire dataset. Finally, the stability of the chosen reference genes was measured again for the whole dataset with geNorm method to cover all the nutrient conditions that were used in this study.

Flavobacterium columnare growth conditions

F. columnare strains B067 and B185 were originally isolated from infected brown trout (*Salmo trutta*) and from rearing tank water during columnaris disease outbreak, respectively, [26] on Shieh medium supplemented with tobramycin [12]. The ancestral Rz bacteria were exposed to lytic phage as described in Laanto *et al.* (2012) to obtain the R colony morphotype [28]. The S colony morphotype appeared spontaneously among the original Rz colonies in a plate culture. The virulence of these different morphotypes of the strain B067 was reported in an earlier study [27]. Throughout this study, Shieh medium with small modifications [47] was used for bacterial cultivations and as a basis of the nutrient-modified media. The bacteria of different colony morphotypes were stored frozen at -80 °C with 10% glycerol and 10% fetal calf serum. For the analyses, the bacteria were revived from the freezer in Shieh medium at +26 ºC under constant shaking (150 rpm) for 48 h to obtain turbid cultures, after which they were used for the experiments. The revived bacterial cultures were enriched in fresh Shieh medium or spread on Shieh agar plates for the studies described below.

Culture conditions of qPCR samples

a) Chondroitinase and collagenase expression in liquid culture

B067 Rz, R and S were cultured in 15–20 ml of liquid Shieh medium. The growth was monitored by measuring the optical density at 570 nm every 0.5–4 hours. After reaching the logarithmic growth phase (5–8.5 hours post inoculation), duplicate samples of 0.5 ml were collected, and the RNA of the samples was stabilized with RNA Protect™ Bacteria Reagent (Qiagen). The samples were forwarded to the RNA isolation step during the same day. Alternatively, 1–1.5 ml of bacterial culture was centrifuged (3 min, 10 000 x g), and the pellet was stored in RNA*later* RNA Stabilization Reagent

(Qiagen) at -20 ºC to be processed later. A minimum of three independent liquid cultures were grown per colony type (see Table S2).

b) Chondroitinase and collagenase expression on agar plate culture

To prepare samples of bacteria grown on a solid surface, diluted overnight culture of B067 Rz, R or S grown in 1x liquid Shieh medium were spread on Shieh agar plates where all the components of the medium were either diluted (with deionized and sterilized water) to 50 % (0.5x), normal 100 % (1x) or concentrated 200 % (2x). After incubating for 48 h at room temperature (+24 °C), bacterial lawn from plates with separate colonies were chosen for RNA isolation. RNA was stabilized and stored for later processing with RNA Protect™ Bacteria Reagent (Qiagen) according to the manufacturer's instructions. From each sample type (colony type-nutrient concentration combination), a minimum of four plates were analyzed (see Table S2).

Sample preparation and RT-qPCR

The total RNA from the liquid and plate culture samples was extracted using an RNeasy Mini Kit (Qiagen) and residual genomic DNA was removed with DNA-free™ (Ambion by Life Technologies) according to the manufacturers' instructions. RNA concentration was measured with a NanoDrop® ND-1000 Spectrophotometer. To ensure that the isolated RNA was intact, the RNA samples were run on an Agilent RNA 6000 Nano Chip in an Agilent 2100 Bioanalyzer platform (Agilent Technologies). RNA integrity numbers (RIN) were analyzed with 2100 Expert Software (Agilent Technologies). Only high-quality RNA was accepted into the following step, resulting in RINs within a scale of 8.3 to 10.0, with a mean of 9.73 (N=75). Qualified samples proceeded to cDNA synthesis immediately after RNA quality validation. Forty ng of DNAse-treated RNA were used as a template in triplicate 20 µl cDNA synthesis reactions, including iScript™ reverse transcriptase in a 1X iScript™ Reaction Mix from an iScript™ cDNA Synthesis Kit (Bio-Rad). cDNA synthesis reactions were incubated under the following conditions: 5 min at 25 ˚C, 30 min at 42 ˚C and 5 min at 85 ˚C. Three replicate cDNA reactions were pooled. Each sample was run with each primer pair in triplicate. Twenty µl qPCR reactions contained 40 ng of cDNA as a template, 0.5 µM of both forward and reverse primers and 1X iQ™ SYBR Green Supermix (Bio-Rad) as a reaction mix, including iTaq DNA polymerase (25 U/ml). The thermocycling conditions for the qPCR reactions were as follows: 95 ˚C for 3 min, followed by 40 cycles of 95 ˚C for 10 s, Tm ˚C for 20 s and 72 ˚C for 20 s, where Tm (melting temperature) was dependent on the primer pair in question (see Table S1). The qPCR runs were performed in a CFX96™ Real-Time System in C1000™ and C1000™ Touch Thermal Cyclers (Bio-Rad) on 96-well Hard-Shell® PCR plates (Bio-Rad). To reduce the variation between plates, two inter-plate calibrator (IPC) samples were run in triplicates on each plate.

Relative expression analysis

Missing Cq (quantification cycle) values were replaced with the average Cq of a sample's qPCR replicates. Cq values were calibrated with IPC (interplate calibrator) samples within each gene and corrected with the efficiency determined specifically for each primer pair. The average Cq of qPCR repeats was calculated, after which the Cq values of the target genes, chondroitinase (*cslA*) and collagenase, were normalized against those of two reference genes, *gapdh* and *glyA*. Finally, the Cq values were

converted into relative gene expression. All qPCR data analyses described above were performed with GenEx v5.3.4 (MultiD Analyses). To maintain a normal distribution, the relative quantities were log-transformed for the statistical analyses.

Sequencing of chondroitinase and collagenase genes

Chondroitinase and collagenase genes, including the upstream regions (approximately 100 bp) assumably covering the promoter regions, were sequenced from *F. columnare* B067 colony types Rz, R and S. The DNA sequences were determined in an automatic sequencer (Applied Biosystems 3130*xl* Genetic Analyzer) with the Sanger sequencing method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing Analysis Software v6.0 (Applied Biosystems) was used to analyze the raw sequencing data, and the gene sequences were aligned to find differences between the colony types using Vector NTI Advance® 11.5.0 (Invitrogen).

Virulence experiments with rainbow trout

For the experimental infections, Rz colony type of *F. columnare* strains B067 and B185 were grown in the conditions described above. The compositions of the growth media were manipulated by altering only the concentration of yeast extract and peptone. The bacteria were cultivated in three different nutrient concentrations, 2xN, 1x or 0.5xN Shieh. N refers to the concentrations of peptone and yeast extract that were doubled, the same or halved (when compared to 1x Shieh), respectively. Detailed composition of the growth media used in this study are presented in Supplementary Table S3.

Apparently healthy rainbow trout (*Oncorhynchus mykiss*) fry with no previous contact with *F. columnare* were obtained from a fish farm in Central Finland in spring, before the warm water season. Prior to the experiments the fish were maintained in aerated ground water at 17 °C in 250-l flow-through aquaria. For the infection experiments the water temperature was gradually elevated to 24 °C during seven days. Rainbow trout fry (n=16 per each treatment, mean weight 0.195 g (n=111)) were placed individually in 0.75-liter aquaria containing 500 ml of ground water (T=24.1 °C). The fish were challenged by adding bacterial culture (7.5-12 µl of original culture diluted in total of 400 µl of Shieh medium) directly into aquaria to reach an infective dose of $5x10^3$ CFU ml^{-1} of *F. columnare.* In this continuous infection, bacteria are present in the aquaria throughout the experiment. Control fish were exposed to sterile 1x Shieh medium (n=15). The fish longevity was monitored in intervals of 30-60 minutes, starting from the addition of bacterial culture. To meet the requirements of ethical endpoint of the experimental animals, morbid fish that did not respond to external stimuli were considered dead and removed from the experiment, and euthanized. To determine the presence or absence of *F. columnare* on the fish, skin cultivations on Shieh agar supplemented with tobramycin [12] were taken from the moribund fish and from the fish that were alive in the end of the experiment.

The fish experiments were conducted according to the Finnish Act on Use of Animals for Experimental Purposes, under permission ESAVI-3940/04.10.07/2015 granted for L-RS by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland.

Statistics

The effects of colony type and nutrient concentration on chondroitinase and collagenase of log-transformed gene expression were analyzed via ANOVA and post-hoc analyses within each colony type were Bonferroni-corrected. Bacterial virulence after culturing in various nutrient conditions was analyzed from rainbow trout longevity data using Kaplan-Meier survival analysis. All statistical analyses were performed with IBM SPSS Statistics 22.

FUNDING INFORMATION

This work was funded by Academy of Finland grants #252411 (the Centre of Excellence in Biological Interactions 2012-2017), #272995 (for L-RS) and Maj and Tor Nessling Foundation. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication in Microbial Ecology.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Annemie Decostere, Ms Irene Helkala, MSc Ville Hoikkala, PhD Elina Laanto, MSc Katja Neuvonen, MSc Marjut Paljakka, Mr Petri Papponen and PhD Ilona Rissanen for assistance in the laboratory.

REFERENCES

1. Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245

2. Arias CR, 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

3. Bernardet JF (1997) Immunization with bacterial antigens: *Flavobacterium* and *Flexibacter* infections. Dev Biol Stand 90:179

4. Bertolini JM, Rohovec JS (1992) Electrophoretic detection of proteases from different *Flexibacter-Columnari*s strains and assessment of their variability. Dis Aquat Org 12:121

5. Brown SP, Cornforth DM, Mideo N (2012) Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends Microbiol 20:336

6. Bruno J, Petes L, Harvell C, Hettinger A (2003) Nutrient enrichment can increase the severity of coral diseases. Ecol Lett 6:1056

7. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE

guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611

8. Casadevall A, Pirofski L (2001) Host-pathogen interactions: the attributes of virulence. J Infect Dis 184:337

9. Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol 13:343

10. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS (2010) ppGpp conjures bacterial virulence. Microbiol Mol Biol Rev 74:171

11. Declercq AM, 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

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

13. Duchaud E, Boussaha M, Loux V, Bernardet JF, Michel C, Kerouault B, Mondot S, Nicolas P, Bossy R, Caron C, Bessieres P, Gibrat JF, Claverol S, Dumetz F, Le Henaff M, Benmansour A (2007) Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. Nat Biotechnol 25:763

14. Dumpala PR, Gulsoy N, Lawrence ML, Karsi A (2010) Proteomic analysis of the fish pathogen *Flavobacterium columnare*. Proteome Sci 8:26

15. Gorke B, Stulke J (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol 6:613

16. Guijarro JA, Cascales D, Garcia-Torrico AI, Garcia-Dominguez M, Mendez J (2015) Temperature-dependent expression of virulence genes in fish-pathogenic bacteria. Front Microbiol 6:700

17. Hall SR, Knight CJ, Becker CR, Duffy MA, Tessier AJ, Caceres CE (2009) Quality matters: resource quality for hosts and the timing of epidemics. Ecol Lett 12:118

18. Harrington DJ (1996) Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. Infect Immun 64:1885

19. Houle MA, Grenier D, Plamondon P, Nakayama K (2003) The collagenase activity of *Porphyromonas gingivalis* is due to Arg-gingipain. FEMS Microbiol Lett 221:181

20. Kassegne K, Hu W, Ojcius DM, Sun D, Ge Y, Zhao J, Yang XF, Li L, Yan J (2014) Identification of collagenase as a critical virulence factor for invasiveness and transmission of pathogenic *Leptospira* species. J Infect Dis 209:1105

21. Ketola T, Mikonranta L, Laakso J, Mappes J (2016) Different food sources elicit fast changes to bacterial virulence. Biol Lett 12:0150660

22. Kunttu HM, Sundberg LR, Pulkkinen K, Valtonen ET (2012) Environment may be the source of *Flavobacterium columnare* outbreaks at fish farms. Environ Microbiol Rep 4:398

23. Kunttu HM, Jokinen EI, Valtonen ET, Sundberg LR (2011) Virulent and nonvirulent *Flavobacterium columnare* colony morphologies: characterization of chondroitin AC lyase activity and adhesion to polystyrene. J Appl Microbiol 111:1319

24. Kunttu HM, Suomalainen LR, Jokinen EI, Valtonen ET (2009) *Flavobacterium columnare* colony types: connection to adhesion and virulence? Microb Pathog 46:21

25. Kunttu HMT, Valtonen ET, Jokinen EI, Suomalainen L (2009) Saprophytism of a fish pathogen as a transmission strategy. Epidemics 1:96

26. Laanto E, Sundberg LR, Bamford JK (2011) Phage specificity of the freshwater fish pathogen *Flavobacterium columnare*. Appl Environ Microbiol 77:7868

27. Laanto E, Penttinen RK, Bamford JKH, Sundberg L (2014) Comparing the different morphotypes of a fish pathogen - implications for key virulence factors in *Flavobacterium columnare*. BMC Microbiol 14

28. Laanto E, Bamford JKH, Laakso J, Sundberg L (2012) Phage-driven loss of virulence in a fish pathogenic bacterium. Plos One 7

29. Lantz M (1997) Are bacterial proteases important virulence factors? J Periodont Res 32:126

30. Lebrun I, Marques-Porto R, Pereira AS, Pereira A, Perpetuo EA (2009) Bacterial toxins: An overview on bacterial proteases and their action as virulence factors. Mini-Rev Med Chem 9:820

31. Li N, Qin T, Zhang XL, Huang B, Liu ZX, Xie HX, Zhang J, McBride MJ, Nie P (2015) Gene deletion strategy to examine the involvement of the two chondroitin lyases in *Flavobacterium columnare* virulence. Appl Environ Microbiol 81:7394

32. Li N, Zhang J, Zhang LQ, Nie P (2010) Difference in genes between a high virulence strain G(4) and a low virulence strain G(18) of *Flavobacterium columnare* by using suppression subtractive hybridization. J Fish Dis 33:403

33. McKenzie VJ, Townsend AR (2007) Parasitic and infectious disease responses to changing global nutrient cycles. Ecohealth 4:384

34. Nakayama H, Tanaka K, Teramura N, Hattori S (2015) Expression of collagenase in *Flavobacterium psychrophilum* isolated from cold-water disease-affected ayu (*Plecoglossus altivelis*). Biosci Biotechnol Biochem 80:135

35. Olivares-Fuster O, Arias CR (2008) Use of suppressive subtractive hybridization to identify *Flavobacterium columnare* DNA sequences not shared with *Flavobacterium johnsoniae*. Lett Appl Microbiol 46:605

36. Olivares-Fuster O, Baker JL, Terhune JS, Shoemaker CA, Klesius PH, Arias CR (2007) Host-specific association between *Flavobacterium columnare* genomovars and fish species. Syst Appl Microbiol 30:624

37. Oogai Y, Matsuo M, Hashimoto M, Kato F, Sugai M, Komatsuzawa H (2011) Expression of virulence factors by *Staphylococcus aureu*s grown in serum. Appl Environ Microbiol 77:8097

38. Ostland VE, Byrne PJ, Hoover G, Ferguson HW (2000) Necrotic myositis of rainbow trout, *Oncorhynchus mykis*s (Walbaum): proteolytic characteristics of a crude extracellular preparation from *Flavobacterium psychrophilum*. J Fish Dis 23:329

39. Pfluger-Grau K, Gorke B (2010) Regulatory roles of the bacterial nitrogen-related phosphotransferase system. Trends Microbiol 18:205

40. Pulkkinen K, Suomalainen LR, Read AF, Ebert D, Rintamaki P, Valtonen ET (2010) Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc Biol Sci 277:593

41. Rao M, Tanksale A, Ghatge M, Deshpande V (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597

42. Revetta RP, Rodgers MR, Kinkle BK (2005) Isolation and identification of freshwater bacteria antagonistic to *Giardia intestinali*s cysts. J Water Health 3:83

43. Rohmer L, Hocquet D, Miller SI (2011) Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. Trends Microbiol 19:341

44. Rudek W, Haque R (1976) Extracellular enzymes of genus *Bacteroides*. J Clin Microbiol 4:458

45. Salyers AA, Kotarski SF (1980) Induction of chondroitin sulfate lyase activity in *Bacteroides thetaiotaomicron*. J Bacteriol 143:781

46. Somerville GA, Proctor RA (2009) At the crossroads of bacterial metabolism and virulence factor synthesis in *Staphylococci*. Microbiol Mol Biol Rev 73:233

47. Song Y, Fryer J, Rohover J (1988) Comparison of six media for the cultivation of *Flexibacter-Columnaris*. Fish Pathol 23:91

48. Stringer-Roth KM, Yunghans W, Caslake LF (2002) Differences in chondroitin AC lyase activity of *Flavobacterium columnare* isolates. J Fish Dis 25:687

49. Sundberg LR, Kunttu HM, Valtonen ET (2014) Starvation can diversify the population structure and virulence strategies of an environmentally transmitting fish pathogen. BMC Microbiol 14:67

50. Sundell K, Wiklund T (2015) Characteristics of epidemic and sporadic *Flavobacterium psychrophilum* sequence types. Aquaculture 441:51

51. Suomalainen LR, Tiirola M, Valtonen ET (2006) Chondroitin AC lyase activity is related to virulence of fish pathogenic *Flavobacterium columnare*. J Fish Dis 29:757

52. Tekedar HC, Karsi A, Gillaspy AF, Dyer DW, Benton NR, Zaitshik J, Vamenta S, Banes MM, Gulsoy N, Aboko-Cole M, Waldbieser GC, Lawrence ML (2012) Genome sequence of the fish pathogen *Flavobacterium columnare* ATCC 49512. J Bacteriol 194:2763

53. Thomas F, Hehemann J, Rebuffet E, Czjzek M, Michel G (2011) Environmental and gut *Bacteroidetes*: the food connection. Front Microbiol 2

54. Tripathi NK, Latimer KS, Gregory CR, Ritchie BW, Wooley RE, Walker RL (2005) Development and evaluation of an experimental model of cutaneous columnaris disease in koi *Cyprinus carpio*. J Vet Diagn Invest 17:45

55. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034

56. Wagner B, Wise D, Khoo L, Terhune J (2002) The epidemiology of bacterial diseases in food-size channel catfish. J Aquat Anim Health 14:263

57. Wakabayashi H (1991) Effect of environmental conditions on the infectivity of *Flexibacter-Columnaris* to fish. J Fish Dis 14:279

58. Wedekind C, Gessner MO, Vazquez F, Maerki M, Steiner D (2010) Elevated resource availability sufficient to turn opportunistic into virulent fish pathogens. Ecology 91:1251

59. Zenobia C, Hajishengallis G (2015) *Porphyromonas gingivalis* virulence factors involved in subversion of leukocytes and microbial dysbiosis. Virulence 6:236

Figures and tables

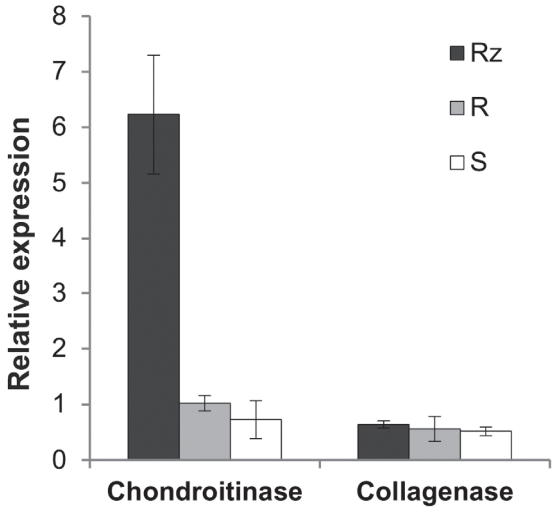

Fig. 1. Gene expression of chondroitinase and collagenase in liquid culture in different colony types. Mean relative gene expression (+/- S.E.) of chondroitinase and collagenase in the virulent Rz and nonvirulent R and S colony morphotypes of *Flavobacterium columnare* B067 grown in liquid 1x Shieh medium.

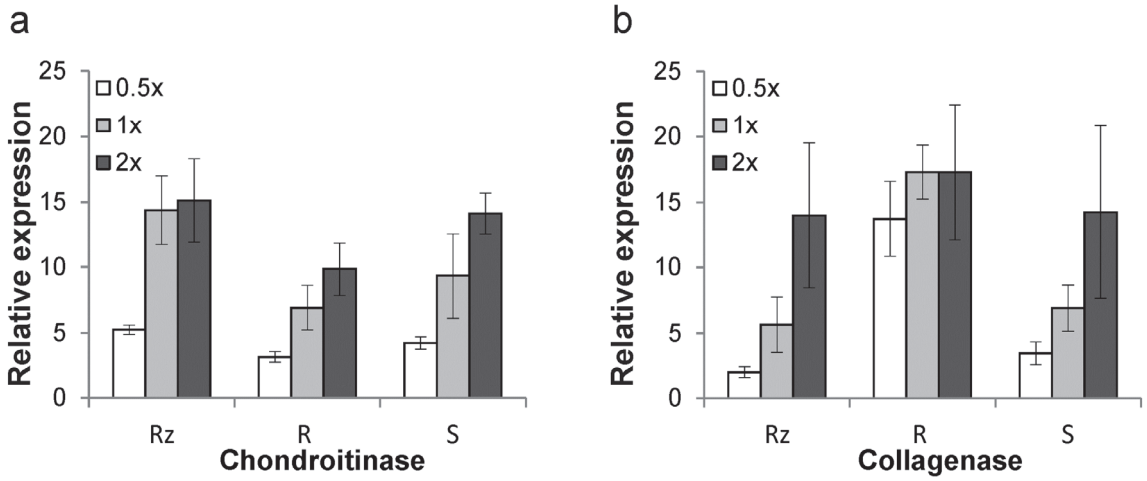

Fig. 2. **Gene expression of chondroitinase and collagenase on agar plates in different colony types.** Mean relative expressions (+/- S.E.) of A) chondroitinase and B) collagenase in Rz, R and S colony morphotypes of *Flavobacterium columnare* B067 grown on diluted (0.5x), normal (1x) and concentrated (2x) Shieh agar. For statistics, see Tables 1 and 2.

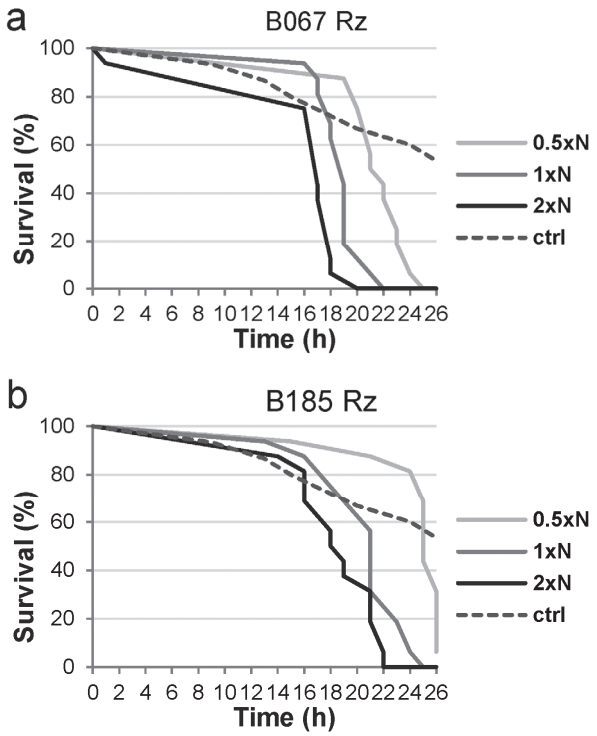

Fig. 3. Effect of nutrient concentration of the bacterial culture on *Flavobacterium columnare* virulence in rainbow trout fry (*Oncorhynchus mykiss*). The fish longevity is measured after experimental infection of *F. columnare* strains A) B067 Rz and B) B185 Rz (final bacterial dose $5*10^3$ CFU ml^{-1} in both experiments). The bacteria used for infections were cultured in 0.5xN, 1x or 2xN Shieh medium (N refers to a modified nutrient concentration). Control fish received 1x Shieh only. Differences in fish mortality between the treatments were significant in all pairwise comparisons ($p \leq 0.006$ for B185 and $p \leq 0.041$ for B067), see full statistics in Table 3.

Table 1. Test statistics for the expression of chondroitinase and collagenase between Rz, R and S colony morphotypes of *Flavobacterium columnare* B067 grown on agar plates under various nutrient concentrations (0.5x, 1x and 2x Shieh). The statistics are given for the overall test and p-values for pairwise post hoc (LSD) comparisons. P-values larger than 0.05 are designated as non-significant (NS).

Overall statistics	**Colony type**			**Nutrient concentration**			**Colony type* nutrient interaction**		
	F	df	p	F	df	p	F	df	p
Chondroitinase	5.094	2	0.010	22.008	2	<0.001	1.618	4	0.186
Collagenase	7.283	2	0.002	5.475	2	0.007	1.323	4	0.276

Post hoc tests (p-values)	**Colony type**			**Nutrient concentration**		
	Rz vs R	Rz vs S	R vs S	0.5x vs 1x	0.5x vs 2x	1x vs 2x
Chondroitinase	0.003	0.046	NS	<0.001	<0.001	0.008
Collagenase	0.001	NS	0.003	NS	0.002	0.030

Table 2. Bonferroni-corrected post hoc comparisons of chondroitinase and collagenase expression within each *Flavobacterium columnare* B067 colony type (Rz, R and S) grown on agar plates with various nutrient concentrations (0.5x, 1x, 2x Shieh). The values given are Bonferroni-corrected p-values for pairwise comparisons in a post-hoc test. P-values which are larger than 0.05 are designated as non-significant (NS).

Gene	Colony type	0.5x vs 1x	0.5x vs 2x	1x vs 2x
Chondroitinase				
	Rz	0.006	0.004	NS
	R	NS	0.011	NS
	S	NS	0.000	0.002
Collagenase				
	Rz	NS	0.002	NS
	R	NS	NS	NS
	S	NS	NS	NS

Table 3. Statistics (Kaplan-Meier survival analysis, log rank Mantel Cox test) of pairwise comparisons of rainbow trout (*Oncorhynchus mykiss*) survival in virulence experiment with two *Flavobacterium columnare* strains (B185 and B067) grown in various nutrient concentrations (0.5xN, 1x, 2xN Shieh). Control fish received sterile 1x Shieh.

***F. columnare* strain**	**Treatment**	**0.5xN**		**1x**		**2xN**	
		χ^2	**p**	**χ^2**	**p**	**χ^2**	**p**
B185 Rz							
	Control	7.612	0.006	13.165	<0.001	14.857	<0.001
	0.5xN			18.512	<0.001	32.160	<0.001
	1x					9.835	0.002
B067 Rz							
	Control	4.191	0.041	8.498	0.004	11.671	0.001
	0.5xN			21.679	<0.001	26.213	<0.001
	1x					4.815	0.028

Supplementary material

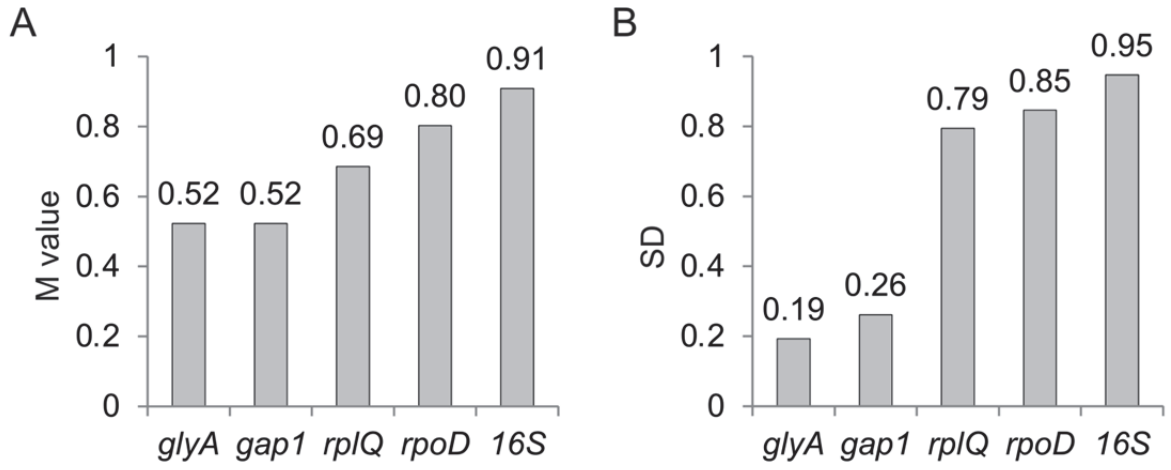

Figure S1. Expression stabilities of candidate reference genes of *F. columnare* B067. Expression stabilities were measured for five genes: *16S rRNA, gapdh, glyA, rplQ* and *rpoD* with two methods: A) M values calculated with geNorm, B) Standard deviation (SD) calculated with Normfinder.

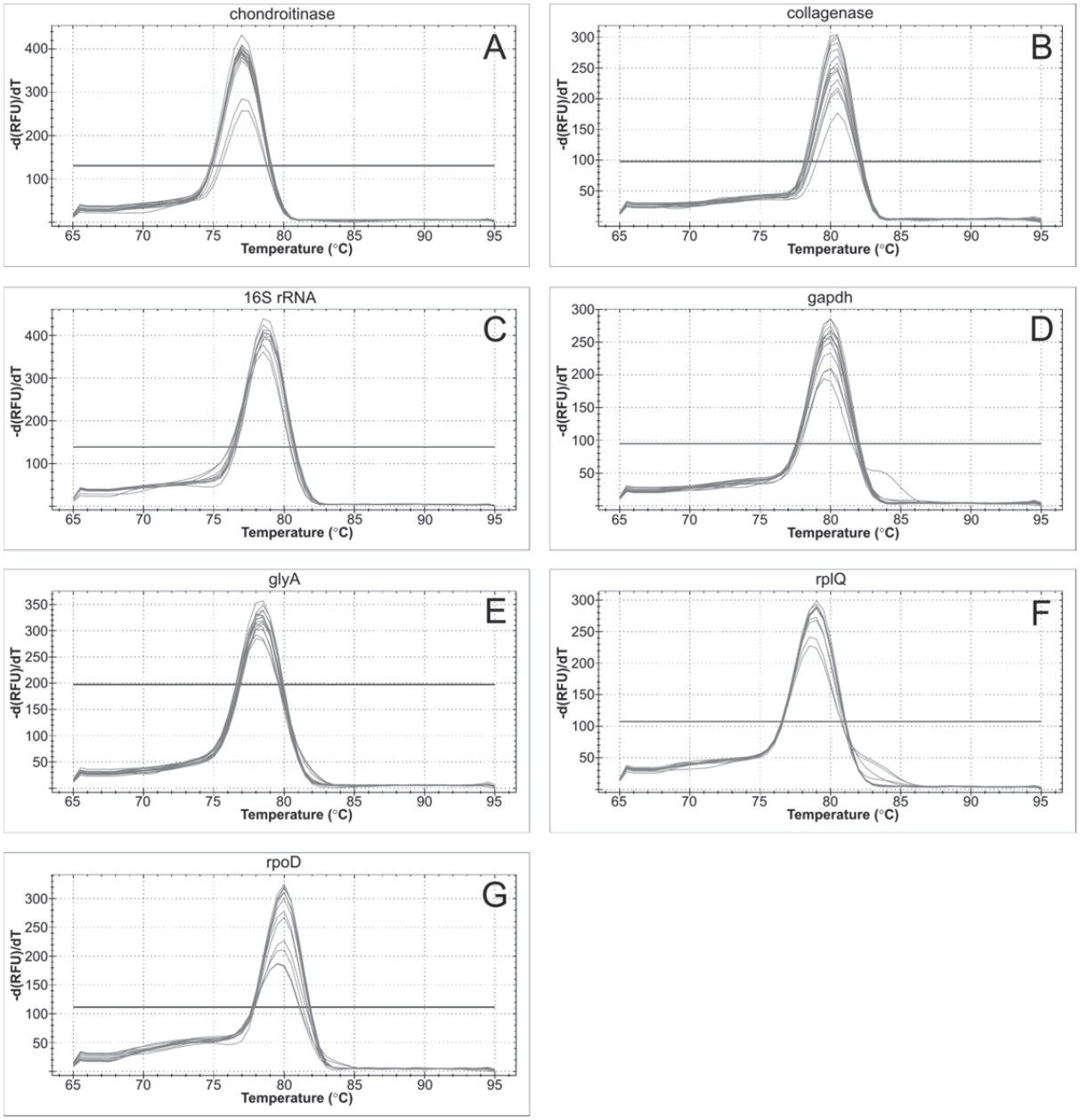

Figure S2. Melt peaks of standard curves for target and reference gene candidates used in the RT-qPCR study of *Flavobacterium columnare*. Melt peaks for A) chondroitinase, B) collagenase, C) *16Sr RNA*, D) *gapdh*, E) *glyA*, F) *rplQ* and G) *rpoD* are provided. One peak denotes one product amplified by the appropriate primer pair.

Table S1. Primer sequences and amplicon details used in RT-qPCR assay for *Flavobacterium columnare*. Amplicon length, Tm (melting temperature), efficiency, R^2, slope and y-intercept are given for a primer pair consisting of a forward (fwd) and a reverse (rev) primer.

Primer name	Sequence (5' - 3')	Amplicon length (bp)	Tm (°C)	Efficiency (%)	R^2	Slope	y-intercept
Primers for target genes							
FC_chond_fwd	CATGCAGGAAATTACGATCCTAAC	146	60	100.0	0.998	-3.323	43.921
FC_chond_rev	GACATTCTATCATGGGCATCTTG						
FC_col_fwd	ACACCAAAGCCTCTTCTGGA	168	60	102.4	0.997	-3.266	43.168
FC_col_rev	CCGTTTTGATGAGCTTGCCA						
Primers for reference genes							
FC_16S_fwd	ACGATCAAACGGCCATTG	119	60	95.4	0.987	-3.438	28.484
FC_16S_rev	AGTAACCTGCCTTCGCAATC						
FC_gap1_fwd	ACCATCCCAAACAGGAGCCGC	98	56	105.7	0.993	-3.193	41.449
FC_gap1_rev	CGTCTGCTGTAGGTACGCGCA						
FC_glyA_fwd	CCAAACCCTTGGGGCTATACAACCC	98	60	102.8	0.999	-3.257	41.479
FC_glyA_rev	AGAGGGCCTCCTTGATTACCTGGAA						
FC_rplQ_fwd	AGCTGCTAAAGTAGGTGACCGTCC	75	56	98.7	0.978	-3.289	45.521
FC_rplQ_rev	GCGTTATCTCCTAAACGGTTCCCCA						
FC_rpoD_fwd	GCGAAGCCGATGTCGTTCGT	108	58	103.3	0.992	-3.245	39.959
FC_rpoD_rev	TTTGACGAACACGTTCGCGGG						

Table S2. Number of *Flavobacterium columnare* B067 replicate liquid cultures and analyzed samples (in brackets) and number of plates sampled for each sample type in RT-qPCR study (Rz, rhizoid; R, rough; S, soft).

	Liquid cultures	Plate samples		
	1x	0.5x	1x	2x
B067 Rz	5 (10)	7	5	6
B067 R	3 (5)	4	6	4
B067 S	5 (9)	8	10	5

Table S3. Compositions of different modified Shieh[1] media used in this study.

Modified Shieh	**1x g/l**	**0.5x g/l**	**2x g/l**	**0.5xN g/l**	**2xN g/l**
$FeSO_4$ x 7 H_2O	0.001	**0.0005**	**0.002**	0.001	0.001
Na-acetate	0.01034	**0.00517**	**0.02068**	0.01034	0.01034
$MgSO_4$ x 7 H_2O	0.3	**0.15**	**0.6**	0.3	0.3
$BaCl_2$ x 2 H_2O	0.01	**0.005**	**0.02**	0.01	0.01
$CaCl_2$ x 2 H_2O	0.0067	**0.00335**	**0.0134**	0.0067	0.0067
$NaHCO_3$	0.05	**0.025**	**0.1**	0.05	0.05
K_2HPO_4	0.1	**0.05**	**0.2**	0.1	0.1
KH_2PO_4	0.05	**0.025**	**0.1**	0.05	0.05
Peptone	5	**2.5**	**10**	**2.5**	**10**
Yeast extract	0.5	**0.25**	**1**	**0.25**	**1**

Set the pH at 7.2
Dilute the components in deionized water. Sterilize by autoclaving.

[1]Song Y, Fryer J, Rohover J. 1988. Comparison of Six Media for the Cultivation of Flexibacter-Columnaris. Fish Pathol **23:**91-94.