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Virulence of *Flavobacterium columnare* genomovars in rainbow trout *Oncorhynchus mykiss*

Jason P. Evenhuis[1,*], Benjamin R. LaFrentz[2]

[1]USDA-ARS, National Center for Cool and Cold Water Aquaculture, 11861 Leetown Rd., Kearneysville, WV 25430, USA
[2]USDA-ARS, Aquatic Animal Health Research Unit, 990 Wire Rd., Auburn, AL 36832-4352, USA

ABSTRACT: *Flavobacterium columnare* is the causative agent of columnaris disease and is responsible for significant economic losses in aquaculture. *F. columnare* is a Gram-negative bacterium, and 5 genetic types or genomovars have been described based on restriction fragment length polymorphism of the 16S rRNA gene. Previous research has suggested that genomovar II isolates are more virulent than genomovar I isolates to multiple species of fish, including rainbow trout *Oncorhynchus mykiss*. In addition, improved genotyping methods have shown that some isolates previously classified as genomovar I, and used in challenge experiments, were in fact genomovar III. Our objective was to confirm previous results with respect to genomovar II virulence, and to determine the susceptibility of rainbow trout to other genomovars. The virulence of 8 genomovar I, 4 genomovar II, 3 genomovar II-B, and 5 genomovar III isolates originating from various sources was determined through 3 independent challenges in rainbow trout using an immersion challenge model. Mean cumulative percent mortality (CPM) of ~49% for genomovar I isolates, ~1% for genomovar II, ~5% for the II-B isolates, and ~7% for the III isolates was observed. The inability of genomovar II isolates to produce mortalities in rainbow trout was unanticipated based on previous studies, but may be due to a number of factors including rainbow trout source and water chemistry. The source of fish and/or the presence of sub-optimal environment may influence the susceptibility of rainbow trout to different *F. columnare* genomovars.

KEY WORDS: *Flavobacterium columnare* · Rainbow trout · Genomovar · Virulence · Immersion challenge

INTRODUCTION

Columnaris disease was initially described by Davis (1922) in fish from the Mississippi River, but the etiological agent *Flavobacterium columnare* was not isolated until 20 yr later (Ordal & Rucker 1944). *F. columnare* is a Gram-negative bacterium that affects many commercially relevant aquaculture finfish species including channel catfish, tilapia, and rainbow trout (Welker et al. 2005, Evenhuis et al. 2014, Shoemaker & LaFrentz 2015). Five different genomovar classifications have been described for *F. columnare* isolates: I, II, II-B, III and I/II, based on the restriction fragment length polymorphism (RFLP) of the 16S rRNA gene (Triyanto & Wakabayashi 1999, Olivares-Fuster et al. 2007a, LaFrentz et al. 2014). Isolates from genomovar I/II were included in an alternative study (Evenhuis et al. 2016) and were not tested here. Currently, all genomovar typed and published *F. columnare* isolates recovered from naturally occurring disease outbreaks in rainbow trout *Oncorhynchus mykiss* have been assigned to genomovar I (LaFrentz et al. 2012b, Evenhuis et al. 2014) except for one, GA-02-14 (Welker et al. 2005), which is a

*Corresponding author: jason.evenhuis@ars.usda.gov

genomovar III isolate (LaFrentz et al. 2014). This may indicate that genomovar I isolates of *F. columnare* are specifically responsible for naturally occurring columnaris outbreaks in rainbow trout and that a host-specific association exists. This phenomenon was also seen in wild threadfin shad *Dorosoma petenense*, in which a host-specific association was suggested between this fish species and genomovar I isolates (Olivares-Fuster et al. 2007b), even though multiple genomovars were present. However, it may also be possible that the other genomovars are not prevalent in regions that predominantly produce rainbow trout. Genomovar II strains were suggested to be more virulent, during laboratory challenges, than genomovar I isolates in multiple fish species, including channel catfish (Shoemaker et al. 2008), zebrafish (Olivares-Fuster et al. 2007b), and rainbow trout (LaFrentz et al. 2012b).

The RFLP technique used to classify *F. columnare* isolates was recently standardized (LaFrentz et al. 2014) and it was discovered that some of the genomovar I isolates used in the aforementioned studies, i.e. in the study of rainbow trout (LaFrentz et al. 2012b) and channel catfish (Shoemaker et al. 2008), were in fact genomovar III isolates. Thus, this finding confounds the virulence differences that were previously observed, and LaFrentz et al. (2014) suggested that the association between virulence and genomovar be re-examined. Shoemaker & LaFrentz (2015) recently determined the virulence of the 5 genomovars in hybrid tilapia *Oreochromis niloticus* × *O. aureus* by immersion challenge, and the results demonstrated a lack of an association between genomovar and virulence in this species under the challenge conditions used. Additional factors, apart from host species and genomovar, can influence *F. columnare* pathogenesis, including water chemistry, within-host species variation, and bacterial growth protocols. Several factors associated with water chemistry will influence both *F. columnare* attachment to gill filaments and mortalities resulting from exposure to *F. columnare,* including the concentration of divalent ions, nitrite, organic matter, and temperature (Declercq et al. 2013, Straus et al. 2015). High salinity has a negative effect on adhesion, i.e. as salinity increases, *F. columnare* adhesion decreases (Altinok & Grizzle 2001). A recent study by Straus et al. (2015) suggests that the hardness of water, specifically the concentration of divalent cations, soft water, or water low in calcium dramatically reduces the number of mortalities in channel catfish immersion challenged with *F. columnare*.

Immersion challenge is the most common protocol for experimental *F. columnare* infection; however, this type of challenge can be influenced by the source water, and Straus et al. (2015) suggested this can lead to difficulties in comparing results between laboratories. Another variable that can influence the results of bacterial challenges is the stock of fish used for testing. LaFrentz et al. (2012a) demonstrated that there is genetic variation in the resistance of channel catfish to *F. columnare*. Similarly, Evenhuis et al. (2015) described variation of innate susceptibility of rainbow trout to *F. columnare* that exists between families of a single line. These studies suggest that depending on the fish used, results can be difficult to reproduce between laboratories and between experiments within a laboratory.

We tested multiple isolates of *F. columnare* from 4 of the 5 described genomovars using a single population of rainbow trout; genomovar I/II was examined in an alternative study (Evenhuis et al. 2016). We show that this population of trout, under these conditions, is predominately susceptible to genomovar I isolates and not as susceptible to other genomovar isolates.

MATERIALS AND METHODS

Fish husbandry

Commercially available, certified disease-free rainbow trout eggs were acquired from Troutlodge, Sumner, WA, USA. Viable hatched trout were hand-fed daily to satiation using a commercially available trout feed (Ziegler). Trout were maintained in flow-through water at a rate of 1 l min^{-1}, at 12.5°C until the challenge weight of ~1.2 g was met. Fish were moved to challenge aquaria 1 wk prior to the immersion challenge to acclimate to an elevated water temperature of 16°C. This procedure was approved under International Animal Care and Use Committee (IACUC) review (protocol #77).

Bacteria culture conditions

A total of 20 *Flavobacterium columnare* isolates from 4 different genomovars (I, II, II-B, and III) (see Table 1) were used in immersion challenges with rainbow trout. Bacterial stocks consisted of individual pure isolates stored at −80°C in 80% tryptone yeast extract salts (TYES) broth (Holt et al. 1994) and 20% glycerol. Bacterial cultures for challenges were

grown as previously described (Evenhuis et al. 2014). Briefly, bacteria were streaked for isolation from stock tubes onto TYES containing 1% agar and grown overnight at 30°C. A single colony was selected to inoculate 10 ml TYES broth and grown overnight at 30°C and 200 rpm in an Innova 44r (New Brunswick Scientific) incubator. These cultures were used to inoculate Fernbach flasks containing 1 l of TYES broth and grown at 30°C and 200 rpm to an optical density of 0.7 to 0.75 at 540 nm. A control flask, which was mock inoculated, was also incubated alongside flasks containing the different isolates.

Immersion challenge protocol

Three independent challenges, each including all 20 isolates, were performed using triplicate tanks for each bacterial isolate, with each tank containing 30 fish between 1.2 and 1.5 g for each isolate tested. Challenges were conducted in 4 l aquaria with restricted water flows (~200 ml min^{-1}) and heated to 16°C. Fish were allowed to acclimate for 1 wk prior to challenge. Water flows were stopped during the immersion challenge, and tanks were inoculated with overnight bacterial culture (for specific isolate challenge concentrations, see Table 1) for 1 h, after which water flows were resumed. The bacterial dose was increased for each successive challenge, in line with the increasing size of rainbow trout used in the challenge. This reflects the authors' observations that rainbow trout appear to become less susceptible to *F. columnare* as they age. The increase in challenge dose was to compensate for this apparent reduced susceptibility. Water samples from each tank (3 samples $isolate^{-1}$) were taken 3 to 5 min post tank inoculation, and 100 µl of 10-fold serial dilutions were plated on TYES agar plates and grown as previously stated. Viable colony forming units (CFU) ml^{-1} were enumerated, and the counts from each replicate tank were averaged to determine CFU ml^{-1} dosage. Control tanks were inoculated with TYES broth, and no *F. columnare* growth was observed on plates. Mortalities were removed and counted daily, and approximately 20% of mortalities were randomly tested by homogenizing gill tissue and streaking on TYES agar plates for confirmation of the presence of *F. columnare*. Genomovar confirmation was determined by enzymatic digestion (*Hae*III) of the 16S rRNA gene as previously described (LaFrentz et al. 2014) (see Fig. A1 in the Appendix). Survivors were euthanized using an overdose of MS-222 (tricaine methane sulfonate), at a concentration of 200 mg l^{-1}.

A fourth challenge was conducted as previously described using a single isolate each of genomovar I (CSF-298-10), genomovar II (LSU) and genomovar III (ARS-1). Bacterial isolates were grown in TYES or in modified Shieh broth (Decostere et al. 1997, LaFrentz et al. 2012b) to ascertain the effect of growth media on the virulence of the isolates tested.

Water analysis

Samples from water used for maintaining fish in the present study at the National Center for Cool and Cold Water Aquaculture (NCCCWA) were collected prior to coming into contact with fish. For comparative purposes, water used for maintaining fish at the Aquatic Animal Health Research Unit (AAHRU) in a previous study (LaFrentz et al. 2012b) were also collected. Samples were analyzed for pH, hardness (mg $CaCO_3$ l^{-1}), dissolved oxygen, ammonia, nitrite, nitrate, calcium, potassium, magnesium, sodium, temperature, and conductivity (mS cm^{-1}). Water samples from the NCCCWA and AAHRU were analyzed by Reliance Laboratories and the Alabama Cooperative Extension System, respectively.

Statistical analysis

Cumulative percent mortality (CPM) graphs were generated using GraphPad Prism v.5 software. p-values were calculated by 1-way ANOVA using Tukey's multiple comparison test (95% confidence interval) where <0.05 was deemed significant.

RESULTS AND DISCUSSION

Three independent challenges were performed using 8 genomovar I, 4 genomovar II, 3 genomovar II-B, and 5 genomovar III isolates (Table 1). All genomovar I isolates displayed a range of virulence between 27 and 92% CPM in each challenge, while no genomovar II isolate produced more than 4% mortality in any of the challenges. The genomovar I isolate PB-06-113-2 produced the highest CPM (between 84 and 92%) in all 3 challenges. FBCC-CC-12K was the most virulent of the genomovar II-B isolates, and resulted in 15% CPM. Two of the genomovar III isolates, GA-02-14 and ALM-05-53, were able to produce mortalities in all 3 challenges, ranging from 5 to 34% CPM (Fig. 1); the GA-02-14 isolate is the only described non-genomovar I strain that has

Table 1. *Flavobacterium columnare* isolates used for 3 independent challenges in rainbow trout *Oncorhynchus mykiss*, including the source of each isolate, genomovar, citation, the colony forming unit CFU used for each challenge, cumulative percent mortality (CPM) with the standard error mean (SEM), approximate fish size, fish age in degree days, and the optical density range at 540 nm (OD_{540}) for each culture when used for challenge

Isolate	Species source	Genomovar	Reference	Challenge I		Challenge II		Challenge III	
				CFU ($\times 10^6$)	CPM (SEM)	CFU ($\times 10^6$)	CPM (SEM)	CFU ($\times 10^6$)	CPM (SEM)
CSF298-10	*Oncorhynchus mykiss*	I	Evenhuis et al. (2014)	1.71	46.7 (4.4)	2.78	40 (10.4)	3.91	42.8 (6.4)
PB-06-113-2	*Micropterus salmoides*	I	Shoemaker & LaFrentz (2015)	2.21	88.3 (4.4)	2.59	84.9 (5.4)	6.2	91.7 (6)
023-08-2	*Oncorhynchus mykiss*	I	LaFrentz et al. (2012b)	1.94	49.7 (17.3)	2.65	33.9 (9.4)	3.7	48.3 (10.1)
031-10-S5	*Oncorhynchus mykiss*	I	LaFrentz et al. (2012b)	1.27	49.4 (9.2)	1.48	51.7 (3.3)	2.02	56.7 (3.3)
030-10-S5	*Oncorhynchus mykiss*	I	LaFrentz et al. (2012b)	1.98	49.0 (3.7)	3.92	56.7 (9.3)	5.55	41.7 (7.3)
ISRAEL	*Cyprinus carpio* (L.)	I	Shoemaker & LaFrentz (2015)	2.88	27.4 (15.0)	3.73	38.3 (8.8)	5.38	46.7 (1.7)
031-10-S1	*Oncorhynchus mykiss*	I	LaFrentz et al. (2012b)	2.22	56.5 (19.0)	3.69	39.2 (12.1)	4.69	45.7 (4.7)
023-08-6	*Oncorhynchus mykiss*	I	LaFrentz et al. (2012b)	2.35	34.8 (5.2)	3.29	41.7 (15.9)	5.47	73.6 (6.2)
AL-02-36	*Micropterus salmoides*	II	Shoemaker & LaFrentz (2008)	1.95	0 (0)	2.97	1.6 (1.6)	4.52	3.3 (1.7)
LSU	*Ictalurus punctatus*	II	Welker et al. (2005)	2.53	0 (0)	4	0 (0)	4.79	1.8 (1.8)
AR-K-CC12K	*Ictalurus punctatus*	II	Shoemaker & LaFrentz (2015)	1.86	1.6 (1.6)	1.83	3.5 (3.5)	3	0 (0)
ALG-00-530	*Ictalurus punctatus*	II	Welker et al. (2005)	3.97	0 (0)	6.8	1.7 (1.7)	10.8	0 (0)
FBCC-CC-12K	*Ictalurus punctatus*	II-B	Shoemaker & LaFrentz (2015)	2.83	15 (5)	3.85	10 (5.8)	3.82	10.6 (3.4)
PT-14-00-151	*Ictalurus punctatus*	II-B	Shoemaker & LaFrentz (2015)	3.4	0 (0)	3.57	0 (0)	7.8	3.4 (1.7)
ALG-00-515	*Ictalurus punctatus*	II-B	Welker et al. (2005)	2.38	3.3 (1.7)	3.5	1.7 (1.7)	4.1	0 (0)
ARS-1	*Ictalurus punctatus*	III	Welker et al. (2005)	2.91	0 (0)	3.83	0 (0)	5.82	1.7 (1.7)
90-106	*Ictalurus punctatus*	III	Shoemaker & LaFrentz (2015)	1.49	0 (0)	1.44	0 (0)	3.51	0 (0)
TN-3-12	*Oreochromis niloticus*	III	Welker et al. (2005)	2.53	0 (0)	3.49	0 (0)	4.36	0 (0)
GA-02-14	*Oncorhynchus mykiss*	III	Welker et al. (2005)	0.53	5 (2.9)	2.68	22 (4.4)	2.66	8.4 (3.3)
ALM-05-53	*Ictalurus punctatus*	III	Shoemaker & LaFrentz (2015)	2.03	29.9 (5.8)	3.79	34.5 (7.6)	2.31	10 (2.9)
			Avg. CFU ($\times 10^6$)	**2.25**		**3.29**		**4.27**	
			~Fish size (g)	1.2		1.2		1.5	
			~Degree days	900		987		1212	
			OD_{540} range	0.7–0.75		0.7–0.75		0.7–0.75	

been isolated from rainbow trout to date (Welker et al. 2005, LaFrentz et al. 2014). The average CPM between genomovars was ~49% for genomovar I, 1% for genomovar II, 5% for genomovar II-B, and 7% for genomovar III (Fig. 1). Previously, the virulence of a genomovar I/II isolate was tested in our laboratory, using similar growth and challenge conditions but with different rainbow trout populations, and a CPM of ~33% was obtained (Evenhuis et al. 2016). The results for the genomovar II isolate in the present study were surprising based on previous work (LaFrentz et al. 2012b) in which 2 genomovar II isolates (ALG-00-530 and LSU) were significantly more virulent to rainbow trout compared to the genomovar I isolate (051-10-S5) and genomovar III isolate (ARS-1) tested, while in this study the genomovar II isolates produced little to no mortalities in challenged trout. It should be noted that the ARS-1 isolate was initially thought to be genomovar I, but was later characterized as a genomovar III isolate following standardization of the RFLP technique (LaFrentz et al. 2014). Three of the isolates used in the previous rainbow trout study (LaFrentz et al. 2012b) were tested here, with only the 051-10-S5 isolate (which we were unable to culture) being replaced with an alternative genomovar I isolate (CSF-298-10). The CPM observed following challenge with ARS-1, ALG-00-530, and LSU were not significantly different than the control fish for any of the 3 challenges. This is in stark contrast to the LaFrentz et al. (2012b) study, where rainbow trout challenged with the genomovar II isolates ALG-00-530 and LSU resulted in greater than 90% mortality, while the ARS-1 isolate resulted in ~20% CPM (LaFrentz et al. 2012b).

One difference between the present study and the previous research of LaFrentz et al. (2012b) was the use of different media for isolate growth. Bacteria grown for the present study were propagated using TYES, while LaFrentz et al. (2012b) used modified Shieh broth. We addressed this difference as we challenged rainbow trout with 3 isolates — one each from genomovar I, II, and III, which were grown in both media — and no differences in CPM were ob-

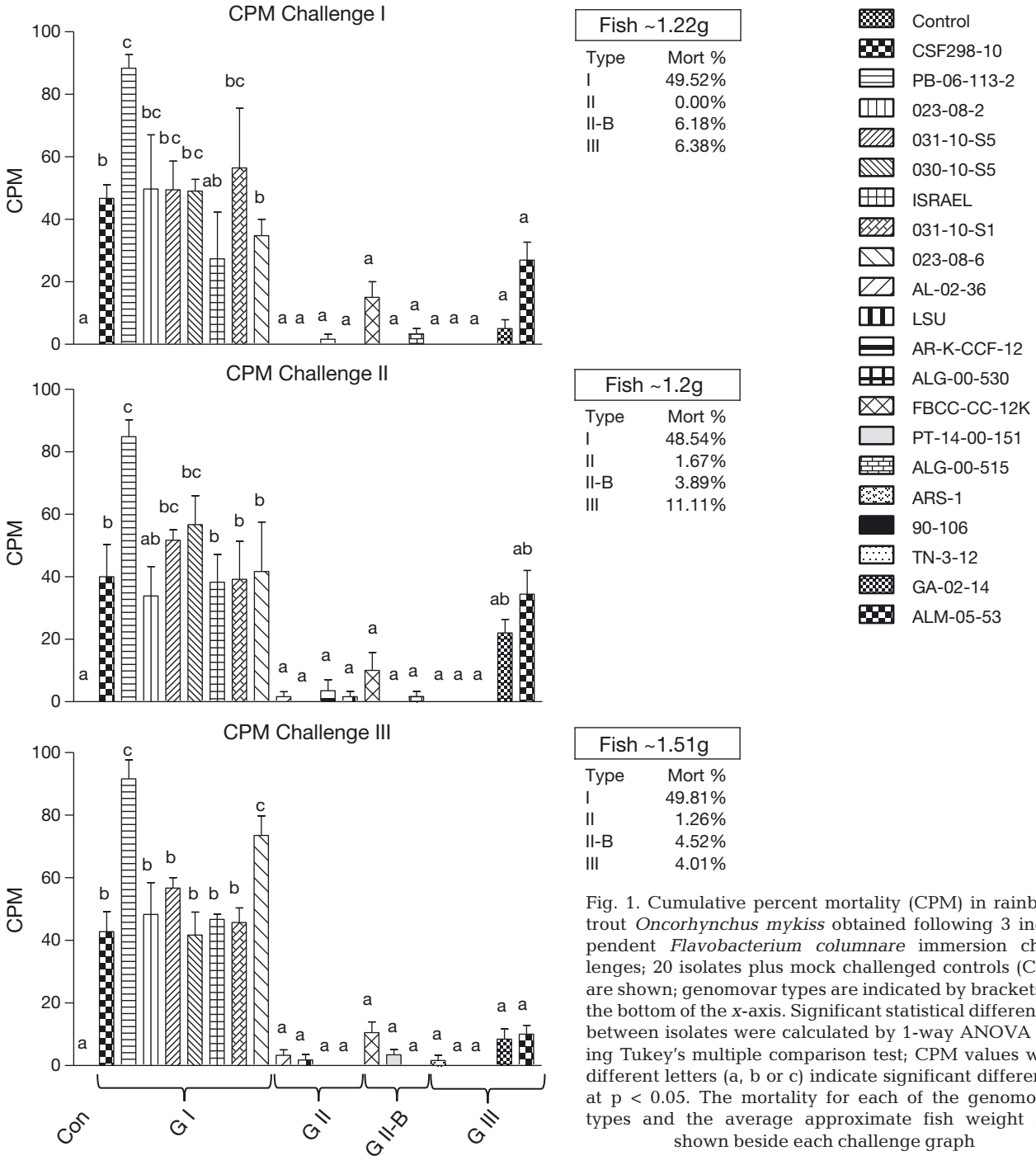

Fig. 1. Cumulative percent mortality (CPM) in rainbow trout *Oncorhynchus mykiss* obtained following 3 independent *Flavobacterium columnare* immersion challenges; 20 isolates plus mock challenged controls (Con) are shown; genomovar types are indicated by brackets at the bottom of the *x*-axis. Significant statistical differences between isolates were calculated by 1-way ANOVA using Tukey's multiple comparison test; CPM values with different letters (a, b or c) indicate significant difference at $p < 0.05$. The mortality for each of the genomovar types and the average approximate fish weight are shown beside each challenge graph

served for the same genomovar grown in the different media prior to challenge (data not shown). This suggests that the difference between genomovar virulence was not a result of the growth media used to propagate each isolate.

Another difference between the LaFrentz et al. (2012b) and the current rainbow trout challenge is the use of different rainbow trout populations. In previous studies, within-line family susceptibility against a single *F. columnare* genomovar I isolate (CSF298-10) ranged from >80 to <10% survival (Evenhuis et al. 2015), indicating that families of rainbow trout within a single breeding line can diverge greatly in *F. columnare* susceptibility.

A third difference between the present study and that of LaFrentz et al. (2012b) is the source of water used for maintaining fish, a factor which has been shown to influence the success of *F. columnare* in

causing disease. The presence of divalent ions, nitrite, water temperature (Decostere et al. 1999, Kunttu et al. 2011), water hardness (Straus et al. 2015), and salinity (Altinok & Grizzle 2001) can influence *F. columnare* adhesion and virulence. Water samples taken during the current study from the NCCCWA were within the optimal range for rainbow trout culture (Table 2), as previously published (Timmons & Ebeling 2010), as were all water parameters tested at the Auburn facility (the LaFrentz et al. 2012b study) except for water hardness. Desired water hardness for salmonid culture is >100 mg $CaCO_3$ l^{-1}, but only 60 mg $CaCO_3$ l^{-1} was measured at the Auburn facility, and by comparision, 298 mg $CaCO_3$ l^{-1} at NCCCWA. Differences in water hardness were attributed as the primary factor that influenced *F. columnare* virulence and gill adhesion in channel catfish between 2 US Department of Agriculture-Agriculture Research Service (USDA-ARS) facilities, Stuttgart National Aquaculture Research Center (SNARC) and the Warmwater Aquaculture Research Unit (WARU) (Straus et al. 2015). The measured hardness at SNARC in which *F. columnare* genomovar II challenges resulted in gill adhesion and mortality, was 128 mg $CaCO_3$ l^{-1}. However, in the water from the WARU in which *F. columnare* genomovar II challenges resulted in low levels of *F. columnare* gill adhesion and no mortality, the measured hardness was only 6.0 mg $CaCO_3$ l^{-1}. The lower hardness levels at the Auburn facility did not preclude successful *F. columnare* challenges in rainbow trout (LaFrentz et al. 2012b), channel catfish (Welker et al. 2005, Shoemaker et al. 2008), or hybrid tilapia (Shoemaker & LaFrentz 2015), regardless of genomovar type. However, the lower hardness level at the Auburn facility may have created a sub-optimal culture environment for rainbow trout, making them more sensitive to the genomovar II isolates. Lower water hardness has been correlated with higher incidence of bacterial kidney disease in rainbow trout (Warren 1963), but determining why lower water hardness would influence host–pathogen interactions for one *F. columnare* genomovar versus another is perplexing. Additionally, differences in bacterial growth and challenge temperature does not influence *F. columnare* virulence, as our lab has grown multiple *F. columnare* isolates at 15 and 30°C prior to challenging at 16°C, and in all cases virulence was not affected (data not shown). LaFrentz et al. (2012b) also grew *F. columnare* isolates at an elevated temperature then subsequently challenged at 16°C and were able to produce mortalities in all *F. columnare* strains tested.

Our results demonstrate that rainbow trout are preferentially susceptible to genomovar I isolates under the conditions tested. This finding, combined with the observation that the majority of published *F. columnare* isolates recovered from disease outbreaks in rainbow trout are genomovar I, may indicate a host-specific association. Previous work investigating *F. columnare* isolated from threadfin shad, freshwater drum, blue catfish, and channel catfish also suggested host-specific associations (Olivares-Fuster et al. 2007b). Genomovar I isolates were predominantly isolated from threadfin shad, while genomovar II isolates were mainly isolated from catfish. Interestingly, all these fish species and *F. columnare* genomovar types could potentially be found in a shared river environment, suggesting a species-specific association. Our results suggest that the virulence of genomovar I *F. columnare* isolates in rainbow trout may be more acute, resulting in natural columnaris outbreaks, or that a host-specific association is occurring. However, it may also be possible that the other genomovars are not prevalent in regions that predominantly produce rainbow trout.

In summary, genomovar I isolates consistently produced mortalities in rainbow trout over 3 independent challenges, though with identical host and environmental conditions,

Table 2. Water sample analysis from the National Center for Cool and Cold Water Aquaculture (NCCCWA) and the Aquatic Animal Health Research Unit (AAHRU) facilities. Hardness as measured in mg $CaCO_3$ l^{-1}, ammonia indicated by total ammonia nitrogen (TAN), and conductivity as mS cm^{-1}. Optimal values for salmonid aquaculture are as described by Timmons & Ebeling (2010). DO: dissolved oxygen; nd: not determined

Parameter	NCCCWA	AAHRU	Optimal values for salmonids
pH	6.74	7.43	6.5–8.5
Hardness (mg $CaCO_3$ l^{-1})	298	60	>100
DO (mg l^{-1})	11	>5	>5
Ammonia (TAN) (mg l^{-1})	0	0	<1.0
Nitrite (mg l^{-1})	0.31	nd	<1 (0.1 in soft water)
Nitrate (mg l^{-1})	4.19	0	0–400
Ca (mg l^{-1})	124	24	4–160
K (mg l^{-1})	2.17	2.6	<5
Mg (mg l^{-1})	11	5.8	<15
Na (mg l^{-1})	5.94	11	<75
Temp (°C)	16	16	nd
Conductivity (mS cm^{-1})	0.47	0.14	nd

while isolates belonging to genomovars II, II-B, and III exhibited a low level of virulence. However, the susceptibility of rainbow trout to other *F. columnare* genomovars may be influenced by the genetics of the rainbow trout population and/or environmental conditions.

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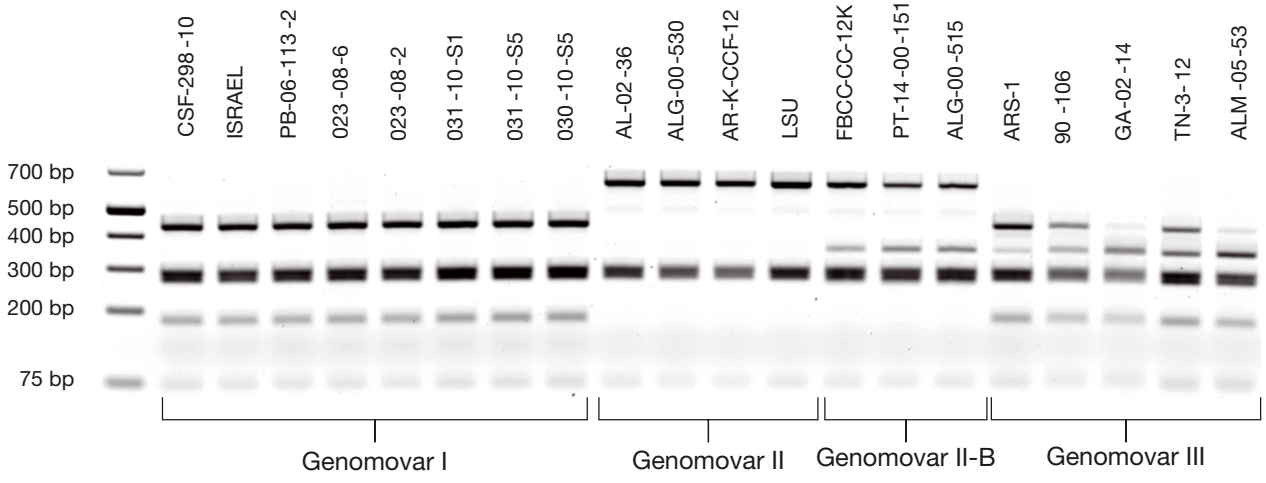
Appendix.

Fig. A1. Restriction fragment length polymorphism gel generated from a PCR of the 16S rRNA gene from each *Flavobacterium columnare* isolate and digested with *Hae*III. Brackets at the bottom of the gel indicate the genomovars, and a 100 kB ladder is shown in the far left lane



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