Virulence and molecular variation of *Flavobacterium columnare* affecting rainbow trout in Idaho , USA

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Abstract

Columnaris disease, caused by *Flavobacterium columnare*, is an emerging problem in the rainbow trout (*Oncorhychus mykiss*) aquaculture industry of Idaho. All *F. columnare* isolates taken from disease outbreaks in the rainbow trout producing region of southern Idaho, and for rainbow trout, are all genomovar I . Virulence phenotyping of 11 genomovar I and 1 genomovar I/II isolates, taken from 6 different farms, suggests significant variation in virulence toward rainbow trout with the most virulent strains having an LD50 of $1x10^6$ CFU/ ml^{-1} when using a waterborne challenge model. The least virulent strain required $8x10^6$ CFU/ ml^{-1} to reach the LD50. AFLP fingerprinting of these strains and the resulting phylogenetic tree show that all of the strains tested had a percent similarity of 75% or higher, save one, the MS-FC-4 strain had less than 60% identity to all of the other strains tested. These results suggest strains of *F. columnare* isolated from disease outbreaks in rainbow trout production have a higher degree of diversity than previously thought.

Keywords: *Flavobacterium columnare*, genomovar I, AFLP, Bacterial challenge

1. Introduction

Flavobacterium columnare was first described by Davis et al. (1922), later isolated in 1944 (Ordal and Rucker 1944) and is the etiological agent for columnaris disease. *F. columnare* is found in aquatic environments worldwide (Decostere et al. 1998; Austin and Austin 1999), and columnaris disease is commonly associated with external lesions on the skin and gills (Decostere et al. 1999; Noga et al. 2000; Thune *et al.* 1993) of finfish in aquaculture production but can also be isolated from internal tissues (Hawke and Thune 1992). Columnaris was estimated to cause up to 60% of bacterial disease loses in the catfish aquaculture industry (Olivares-Fuster *et al.* 2007a; Decostere *et al.* 1997, 1998; Thomas-Jinu et al. 2004) and results in millions of dollars of losses. Recently, columnaris disease has been characterized as an emerging problem for early-life stage rainbow trout (LaFrentz *et al.* 2012; Evenhuis *et al.* 2014).

F. columnare harbors a significant instraspecies diversity and it has been divided into three genomovar (designated I, II and III) based on DNA:DNA hybridization studies (Triyanto and Wakabayshi 1999). For routine typing, genomovar ascription can be carried out by restriction fragment length polymorphism (RFLP) of the 16S rDNA gene (Triyanto and Wakabayshi 1999). Recent studies by LaFrentz *et al.* (2013) and Olivares-Fuster *et al*. (2007a) have increased the number of subdivisions to include I-B, II-B and I/II genomovars by improving on the original RFLP assay.

A study by Olivares-Fuster (2007b) suggested an association between host species and *F. columnare* genomovar where genomovar I isolates were commonly found in threadfin shad (*Dorosoma petenense*) and genomovar II isolates were found in channel catfish and fresh water drum. In that study, all fish species sampled shared the same ecosystem but there was a significant correlation between fish species and which genomovar they harbor. To date, all *F. columnare* isolates taken from rainbow trout outbreaks have been classified as genomovar I, except for one genomovar III isolate from Georgia GA-02-14 (Welker *et al*. 2005). A study describing the emergence of early life stage columnaris disease

outbreaks in Idaho (Evenhuis *et al.* 2014) suggested the expansion of high virulent genomovar I strains in trout aquaculture. Over 70 isolates were tested by 16S rDNA RFLP, multilocus sequence typing and SDS-PAGE analysis. Only one banding pattern difference was observed by SDS-PAGE. In this study we take a closer look at the molecular make up and virulence phenotype of 11 additional *F. columnare* isolates taken from 5 different rainbow trout farms in the Idaho region, including the farm previously described by Evenhuis et al. (2014), as well as the alternative genomovar I/II strain isolated from yellow perch in Indiana. Herein we describe difference in virulence phenotypes, variations in genome fingerprints and differentiation of a targeted gene. This was done to define genetic and virulence variations amongst the *F. columnare* isolates taken from the aquaculture region of Idaho, isolates that were thought to be the expansion of a closely related, highly successful group of *F. columnare*.

2. Method and Materials

2.1 *Fish Husbandry*

Rainbow trout specific-pathogen-free eyed eggs were acquired from Troutlodge Inc., Sumner, WA, USA, or from the even year breeding line at the national center for cool and cold water aquaculture (NCCCWA) (Leeds et al. 2016). Hatched trout were hand fed daily to satiation using a commercially available trout feed (Ziegler Inc., PA) and maintained in flow through water at approximately 12°C , till they were ~2 g in size (Evenhuis *et al.* 2014). All fish use was approved under institutional animal care and use committee review.

2.2 *Bacterial isolates*

All of the genomovar I isolates were obtained from diseased rainbow trout or water from raceways harboring diseased fish, while the genomovar I/II isolate (LaFrentz et al. 2014) was taken from yellow perch (Table 1). *Flavobacterium columnare* isolation was conducted according to a published

protocol (Decostere et al. 1997), on tryptone yeast extract salts (TYES) agar (Holt *et al*. 1988) with or without 1 µg ml^{-1} tobramycin and incubated overnight at 30°C. A total of 12 isolates were characterized by virulence phenotyping and molecular typing (Table 1) and stored at -80°C as 20% glycerol stocks. For challenge, isolates were grown on TYES agar plates with no antibiotics overnight at 30°C followed by selection of a single colony for inoculation of 10 ml of TYES broth. This culture was then incubated overnight at 30°C with constant agitation at 200 rpm in an Innova 44r (New Brunswick Scientific, USA) incubator. A total of 5 µl was used to inoculate a 2800 ml Fernbach flask containing 1 L of TYES, without antibiotics and again grown overnight at 30 C. Cultures were allowed to grow till an OD540 reading between 0.7 and 0.75 was reached. These cultures were then used for immersion challenges.

2.3 *Fish challenge*

All fish were challenged in triplicate 4 L tanks, containing 30 fish per tank, with ~15.8°C water flowing at 200 ml/min and were allowed to acclimate for 7 d prior to challenge. The immersion challenge protocol used was modified from LaFrentz *et al.* (2012). The challenges were performed in 3 L of water for 1 hr statically. The challenge was performed 3 times with 2 different rainbow trout populations (two challenges were done using the NCCCWA even year population and one using a Troutlodge population (Supplemental Table 1)). Bacterial concentrations were estimated by direct plate counting of the challenge water to estimate total CFU the fish were exposed to. Mortalities were removed and recorded daily for 21 d. Gills were sampled from approximately 20% of the daily mortality and were tested for the presence of *F. columnare* by direct plating onto TYES agar plates. Cumulative percent mortality (CPM) data was generated by 1-way ANOVA with Tukey's test for pairwise comparisons and graphs were produced using GraphPad Prism v5 software.

LD50 determination was accomplished using the method of Reed and Muench (1938). Serial 2-fold dilutions of stock *F. columnare* were used to challenge triplicate tanks, and repeated 3 times.

Challenge water was plated onto TYES agar plates and CFUs were visually enumerated. The highest bacterial concentration was 2.75 x 10^7 CFU/ ml^{-1} to a low of 1 x 10^5 CFU/ ml^{-1}.

2.4 *Genomovar characterization*

Genomovar classification was determined using a published protocol (Evenhuis *et al.* 2014). Briefly, total DNA was isolated for Gram-negative bacteria using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions, and concentrations were measured using a NanoDrop ND-1000 spectrophotometer. The 16S rDNA was amplified from total genomic DNA using primer mix (UN-20 (5'- AGAGTTTGATC(AC)TGGCTCAG-3') and R1438 (GCCCTAGTTACCAGTTTTAC)) at a final concentration of 200 µM, 45 µl of Platinum PCR SuperMix High Fidelity polymerase master mix (Invitrogen) and 100 ng of DNA were combined, and the PCR reaction was run on a DNAengine thermocycler (Bio-Rad). The thermocycle program was as follows; 10 m initial 94°C denaturing step, followed by 30 cycles at 94°C, 55°C annealing and 72°C elongation. Each step was 1 m in length. The RFLP profile was generated by digestion of the 16S rDNA product with the *Hae*III or *Dnp*II restriction enzymes and by running the product on a tris-acetate-EDTA (TAE) + 1% (w/v) agarose gel cast with 1x SYBR Safe DNA gel stain (Invitrogen) and visualization by ultraviolet trans illumination.

2.5 *Amplified fragment length polymorphism (AFLP)*

Amplified fragment length polymorphism (AFLP) fingerprinting was performed as previously described (Arias *et al.* 2004; Olivares-Fuster *et al.* 2007). Briefly, 100 ng of genomic DNA was digested using 10 units of *Hind*III and *Taq*I (Promega, Madison, WI) restriction enzymes, in a final volume of 30 µl. Adaptors were ligated, following complete digestion, using the T4 DNA ligase to a final concentration of 0.04 µM for the *Hind*III adaptor and 0.4 µM for the *Taq*I adaptor. The two primers used for AFLP reactions and the PCR conditions are described elsewhere (Olivares-Fuster *et al.* 2007). The *Hind*III primer was labeled with an IR700 fluorochrome from LI-COR (LI-COR, Lincoln, NE). The final products

were separated by electrophoresis on a NEN Global Edition IR2 DNA Analyzer (LI-COR) according to the manufacturer's instructions. Conversion, normalization, background subtraction with mathematical algorithms, and levels of similarity between fingerprints were calculated for AFLP analysis with the Pearson product moment correlation coefficient. Unique ALFP profiles were defined at 95% similarity or lower based on previous studies on the reproducibility of the method.

3. Results

3.1 *Isolate characterization*

Twelve isolates from 5 different farms, 4 from Idaho and 1 from Indiana, were selected based on differences in source tissue, SDS-PAGE patterns, 16S-rDNA restriction patterns (*Dnp*II), and geographical location (Table 1). An alternative SDS-PAGE banding pattern was previously demonstrated by our lab (Evenhuis et al. 2014) and two alternative 16S-rDNA restriction digestion patterns, using *Dnp*II, gave additional bands at approximately 450bp (CSF-485-12-2; CSF-284-13-1) or 250bp (MS-FC-7) (Figure 1). The isolate F10-HK-A was isolated from perch from Indiana and gives a unique 16S-rDNA *Hae*III digestion pattern that has been previously described by LaFrentz *et al.* (2014) and given a genomovar designation of I/II (Figure 1). All of the genomovar I isolates described here are from Idaho rainbow trout.

3.2 *Bacterial challenges and LD50 determination*

All twelve isolates were tested for virulence in rainbow trout and the MS-FC-4 strain produced the highest CPM (94.0% ±2.9) but this was not significantly different from FC-CSF-16 (76%±8.1), FC-CSF-20 (62.8%±8.5), FC-CSF-43 (72%±8.0), CSF-301-12 (61.1%±10.7) or CSF-298-10 (69.7%±4.3). The least virulent strain based on CPM was CSF-253-12 (15.7% ±4.1) but this strain was not significantly different from CSF-284-13-1 (56.4%±8.6), MS-FC-7 (27.8%±5.4), or F10-HK-A (32.8%±4.5) (Figure 2A). Though

most genomovar I strains displayed a cumulative percent mortality greater than 50%, 4 strains had a cumulative percent mortality less than 40%: CSF-253-12, CSF-485-12-2, MS-FC-7 and the genomovar I/II strain F10-HK-A (Figure 2A).

The LD50 was determined for 4 strains, two with high cumulative percent mortality (MS-FC-4 and CSF-298-10) and two with low cumulative percent mortality (CSF-253-12 and MS-FC-7). The two strains with high CPM virulence phenotypes had LD50s of 1x 10^6 CFU/ ml^{-1}, while the low CPM virulence phenotype strains had LD50s of 5x10^6 (MS-FC-7) and 8x10^6 (CSF-253-12) CFU/ ml^{-1} (Figure 2B).

3.3 *AFLP analysis*

All of the strains tested by AFLP analysis in this study had a similarity of approximately 75% or greater, except for the MS-FC-4 strain with a similarity below 60%. Strains FC-CSF-43, CSF-301-13, CSF-298-10, FC-CSF-16, and FC-CSF-20 shared profiles that were 98% similar or higher and thus were considered undistinguisable by AFLP typing.% (Figure 3C). The MS-FC-4 strain showed the most dissimilar AFLP banding profile (lane#12, Figure 3B), missing many of the large/high molecular weight bands at the beginning of the gel compared to all other strains and had the most virulent phenotype, based on the CPM but was not different in its LD50 compared to the CSF-298-10 strain. Interestingly, the genomovar I/II strain F10-HK-A was not the most divergent and most closely clustered with the CSF-485-12-2 and CSF-285-13-1 strains.

4. Discussion

Herein we describe virulence phenotype and molecular variations between 12 individual *F. columnare* isolates, 11 of which were from naturally occurring disease-outbreaks in rainbow trout in Idaho. The twelfth strain was taken from yellow perch columnaris outbreak in Indiana and is classified as genomovar I/II (LaFrentz et al. 2014).

4.1 *Isolate characterization*

Genomovar classification of *F. columnare* isolates have been based on the RFLP pattern of the 16S rDNA when digested with the restriction enzyme *Hae*III but the use of *Dnp*II produces 3 alternative RFLP patterns for the same gene amongst isolates previously classified as genomovar I. This suggests that genetic variation for the 16S rDNA is not completely identifiable with a single RFLP profile and that the diversity of *F. columnare* within each genomovar classification is more complex (LaFrentz *et al.* 2013; Olivares-Fuster *et al.* 2007a) than originally thought (Triyanto and Wakabayshi 1999). Future studies developing a direct typing system via serotyping or multiplex PCR could be used to improve isolate discrimination for in-the field and laboratory studies. We do not suggest making additional genomovar classifications here but future studies classifying isolates on a whole genome basis (AFLP fingerprinting), MLST, or by serotyping could improve the current classification system.

4.2 *Bacterial challenges and LD50 determination*

Within genomovar I, virulence variation in rainbow trout was initially described by LaFrentz et al. (2012) but only 3 isolates from two tissue sources were tested. The LD50 for these isolates was not determined and in-depth molecular characterization, beyond genomovar classification, was also not investigated. Here we show, based on the CPM that virulence phenotypes can vary between 97% to 15% mortality when fish are exposed to similar numbers of bacteria (Figure 2A, Supplemental Table 1). The variation in virulence is more accurately determined when examining LD50 results as the more virulent strains, MS-FC-4 and 298-10-1, can be up to 8 times more virulent than the least virulent strain tested, CSF-253-12 (Figure 2B). The results indicated that virulence variations can occur between isolates taken from similar tissues and/or farms.

Variations in virulence within genomovars was looked at in channel catfish fry (Shoemaker *et al.* 2008) using both genomovar I and II isolates. Cumulative percent mortalities ranged between 60% and

88% for genomovar II isolates and between 0% and 46% for genomovar I isolates. A study using hybrid tilapia (Shoemaker and LaFrentz 2015) comparing virulence between 4 genomovar I isolates resulted in 0% CPM with 3 strains and 100% for the 4th. The same study showed that virulence within genomovar II and genomovar III strains varied from 3% to 78% CPM and 3% to 75% CPM, respectively. The strains used in this study came from much broader selection of sources including many different farms in different geographical regions that were culturing different species of fish.

4.3 *AFLP analysis*

Fingerprint analysis of the 12 isolates used in this study exhibited a high amount of genomic similarity with 6 strains more than 90% similar based on the cluster analysis of their AFLP banding patterns. Five of the other strains were greater than 70% similar to all of the other strains tested (Figure 3). The least similar of the strains tested here, MS-FC-4, scored slightly better than 50% similarity to the rest of the strains. This strain was also one of the most virulent strains tested. Interestingly, the genomovar I/II strain, FK10-HK-A, had higher similarity, approximately 80%, to the all of the strains tested in this study .

In previous studies, grouping isolates between genomovars was cut off at 85% similarity (Arias et al. 2004) so that any isolate displaying less than 85% similarity was considered a different genetic cluster. Only 6 of our isolates achieved greater than 85% similarity to one another (FC-CSF-16, FC-CSF-20, FC-CSF-43, CSF-298-10, CSF-301-13 and MS-FC-7). This suggests that the isolates obtained from the rainbow trout aquaculture region in southern Idaho are not clonal and harbor intrinsic genetic diversity.

5. Conclusion

F. columnare isolates from the rainbow trout producing region of southern Idaho are currently genomovar I and have the typical yellow pigmented, rhizoid colony morphology. Variation in virulence,

AFLP fingerprinting and specific gene sequencing have illustrated a higher amount population heterogeneity than previously shown. This study shows the importance of further phenotypic and molecular testing to accurately characterize disease associated isolates and highlight the diversity that is present in a particular geographic region. Better understanding of strain diversity is extremely important in the development of vaccines or other disease control strategies.

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Table 1. Isolate identification and description including the time, location, and tissue source of isolation. Also any descriptive notes and any previous citations.

Figure 1. The RFLP profiles generated by 16S rDNA, *Hae*III or *Dnp*II, restriction-enzyme digestion and resolved on a 1% TAE agarose gel. M = 100bp marker and the relative (bp) length, (←) = the alternative bands. All isolates from Idaho are genomovar I and the F10-HK-A, from Indiana, is a genomovar I/II based on this analysis.

Figure 2. A. Cumulative percent morality graph and statistical significance $P < 0.05$, produced using GraphPad Prism v.5 and 1-way ANOVA analysis of variance by Tukey test, comparing each *F. columnare* isolate against rainbow trout. Standard error of mean (SEM) are illustrated by error bars, significance is indicated by a, b, c and/or d, multiple letters indicates results are not significantly different from more than one group. B. Calculated LD50 for the CSF-298-10, CSF-253-12, MS-FC-4 and MS-FC-4 strains.

Figure 3. A. List and lane assignment for the AFLP gel in B. C. The phylogenetic tree based on the relatedness of the fingerprint profile illustrated in B.

Figure 1.

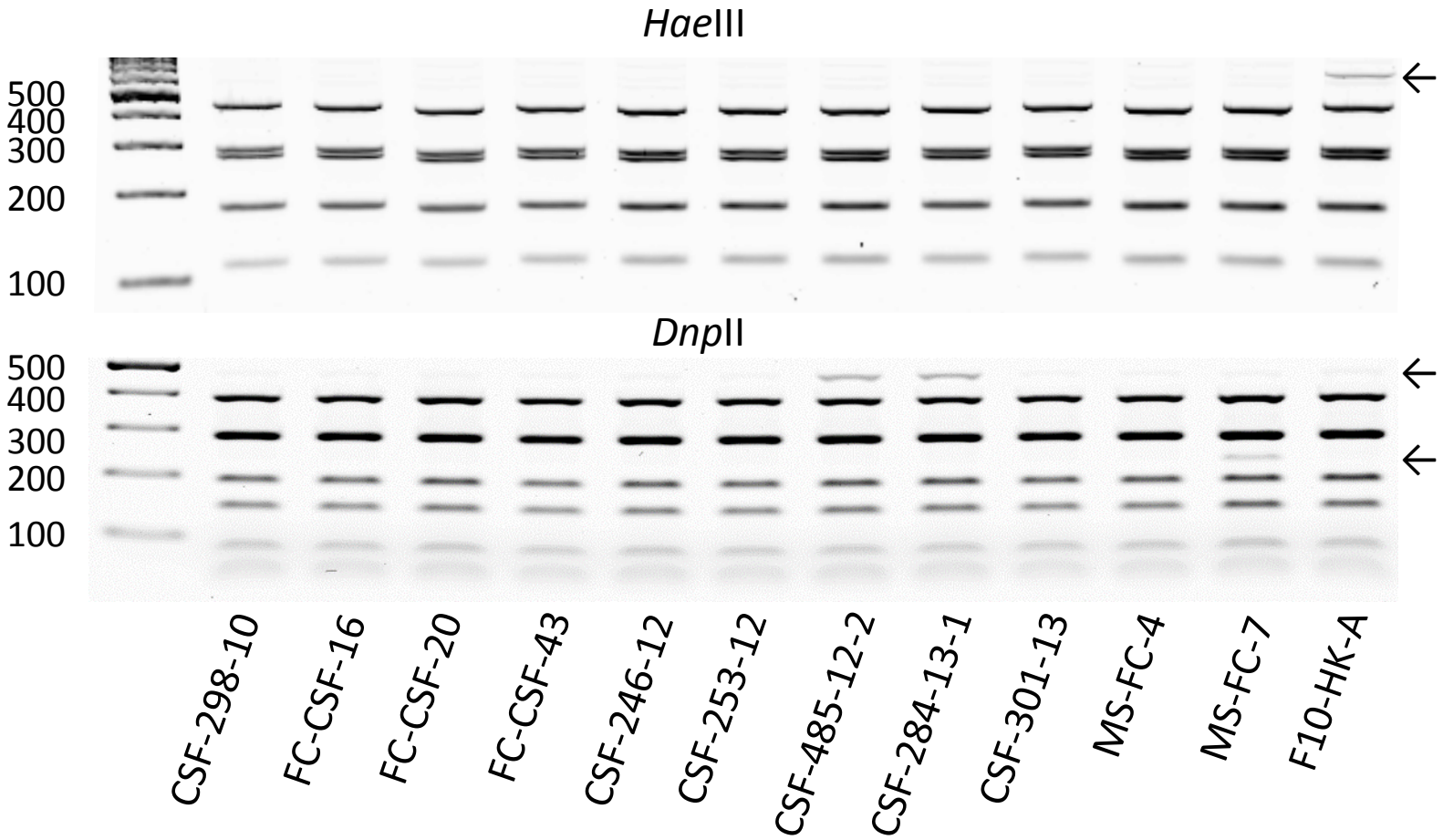
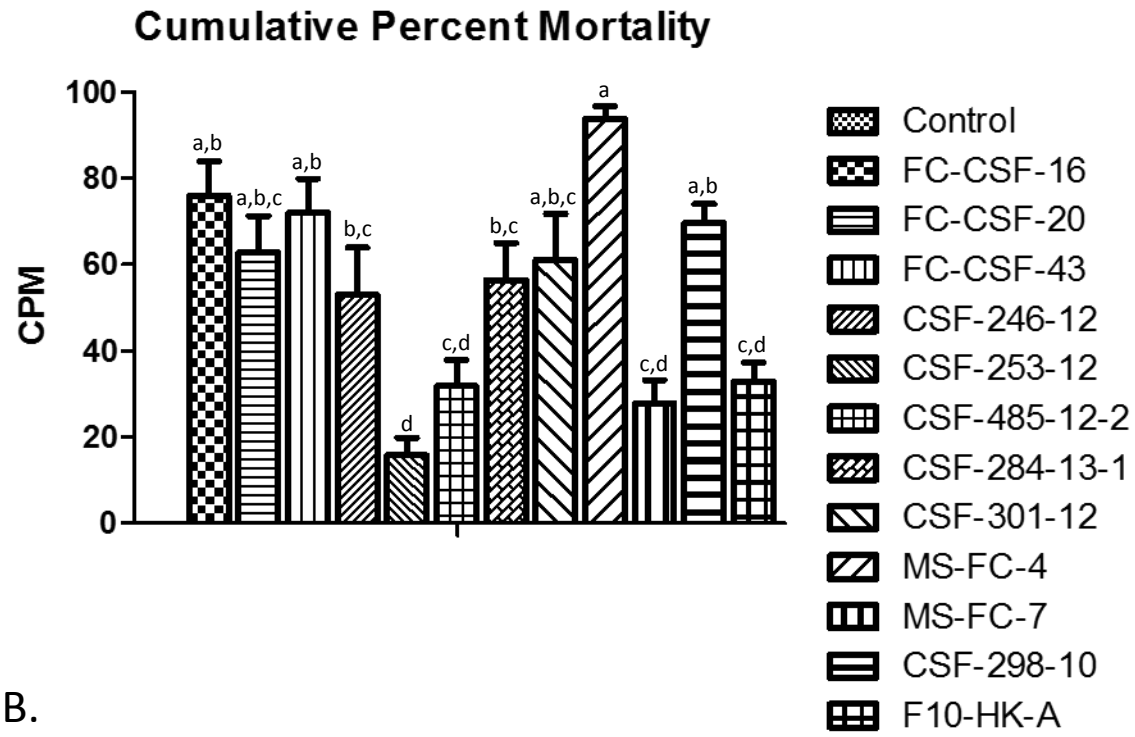

*Hae*III
500
400
300
200
100
*Dnp*II
500
400
300
200
100
CSF-298-10
FC-CSF-16
FC-CSF-20
FC-CSF-43
CSF-246-12
CSF-253-12
CSF-485-12-2
CSF-284-13-1
CSF-301-13
MS-FC-4
MS-FC-7
F10-HK-A

Figure 2.

A.



B.

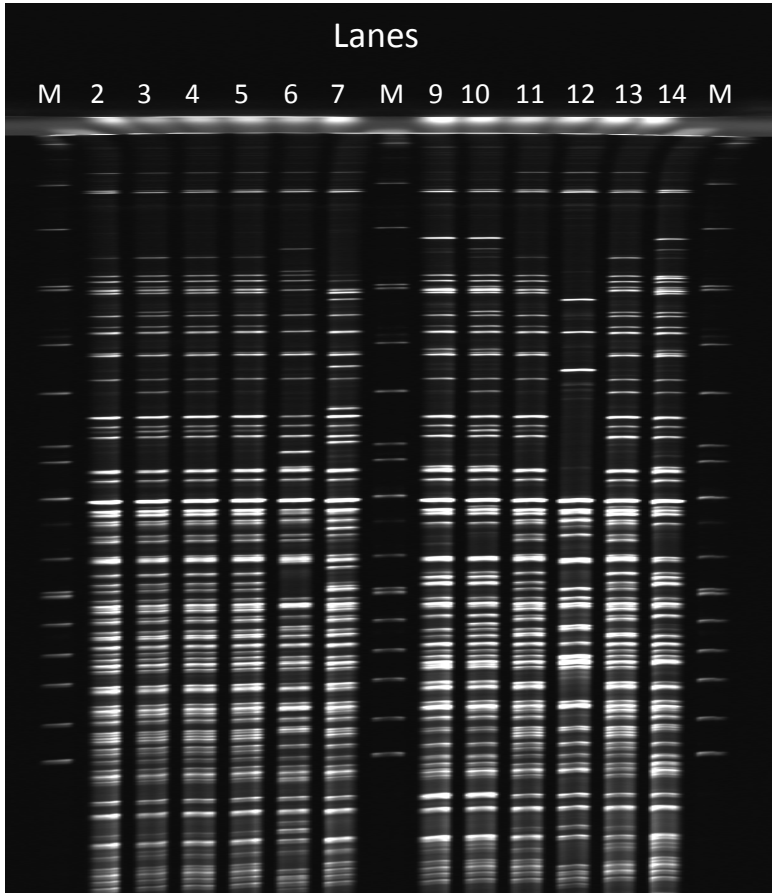
Strain	LD50 CFU x10^6
CSF-253-12	8
CSF-298-10	1
MS-FC-4	1
MS-FC-7	5

Figure 3.

A.

Lane #	**ID**
1	Marker
2	CSF-298-10
3	FC-CSF-16
4	FC-CSF-20
5	FC-CSF-43
6	CSF-246-12
7	CSF-253-12
8	Marker
9	CSF-485-12-2
10	CSF-284-13-1
11	CSF-301-13
12	MS-FC-4
13	MS-FC-7
14	F10-HK-A
15	Marker

B.



C.

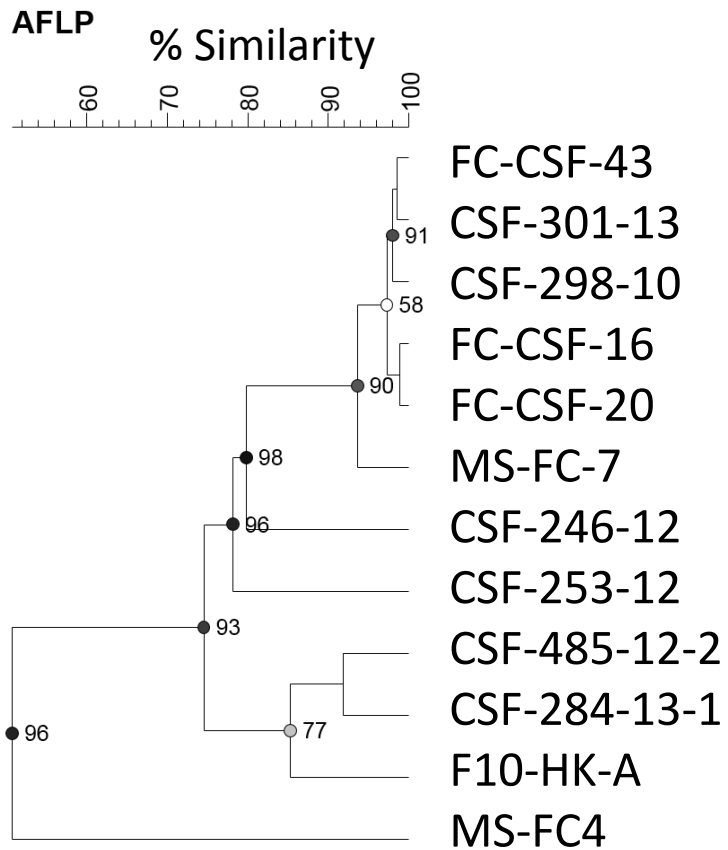


Table 1.

ARS ID	Isolation Date	Location	Tissue	Notes	Citation
CSF-298-10	10/13/2011	CSF - Box Canyon	head kidney	standard challenge strain	Evenhuis et al. 2014
FC-CSF-16	4/2/2012	CSF - Box Canyon	spleen	spleen	This paper
FC-CSF-20	4/2/2012	CSF - Box Canyon	brain	brain	This paper
FC-CSF-43	6/21/2012	CSF - Box Canyon	water	water	This paper
CSF-246-12	6/13/2012	CSF - Clear Lake	gill	alternative SDS PAGE pattern	This paper
CSF-253-12	6/13/2012	CSF - Briggs Creek	gill	Briggs Creek	This paper
CSF-485-12-2	12/3/2012	CSF - Clear Lake	gill	alternative *Dnp*II pattern	This paper
CSF-284-13-1	7/22/2013	CSF - Clear Lake	gill	Clear Lake	This paper
CSF-301-13	8/13/2013	CSF -Box Canyon	kidney	kidney	This paper
MS-FC-4	7/18/2013	Magic Springs	head kidney	alternative SDS PAGE pattern	This paper
MS-FC-7	7/18/2013	Magic Springs	gill	Magic Springs	This paper
F10-HK-A	6/6/2012	Indiana	head kidney	Genomovar I/II, Perch	LaFrentz et al. 2013