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Determination of *Flavobacterium columnare* Virulence Factors in Zebra Fish

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Abstract

Flavobacterium columnare is a gram negative rod that is the cause of Columnaris disease in a variety of fish including ecologically and economically important species in Wisconsin. Virulence is highly strain dependent, as is suitability of the bacteria to genetic manipulation. Infection assays were conducted using a zebra fish (*Danio rerio)* model to identify virulence factors in *F. columnare* using two wild type bacterial strains known to be virulent in channel catfish, C#2 and 94-081.[15,19] To assess the importance of gliding motility and digestive enzymes on bacterial virulence, zebra fish were exposed to solutions containing wild-type *F. columnare* (C#2), a mutant deficient in gliding motility (*gldJ*), or a mutant deficient in chondroitin AC lyase (*clsA*). The loss of either the *clsA* gene or the *gldJ* gene reduced virulence approximately 4-fold. In order to expand these findings, more mutants will need to be generated. A recently developed system for the generation of unmarked deletion mutants in other Flavobacteria requires the use of spontaneous streptomycin resistant mutants. The *F. columnare* wild type 94-081 and streptomycin resistant mutants derived from it are currently being tested in zebra fish to determine virulence levels. Preliminary data suggest that two streptomycin resistant mutants are slightly less virulent than the 94-081 wild-type strain, but similar in virulence to the C#2 wild type strain in zebra fish, indicating that these mutants can be used for future unmarked mutant generation experiments.

Keywords: **Virulence, Mutant, Zebra fish**

1. Introduction

Flavobacterium columnare is an aerobic gram negative rod that is found in aquatic environments. It is the causative agent of Columnaris disease in a variety of freshwater fish species[1]. Columnaris disease results in widespread economic loss in the United States aquaculture industries every year. A number of potential factors contributing to *F. columnare* virulence have been proposed, including motility, adhesion, and the secretion of digestive enzymes[5]. Like many members of the phylum *Bacteroidetes, F. columnare* moves over surfaces by gliding motility, which may allow the bacteria to spread and adhere to lesions in the host organism. Bacteria with a functional motility system exhibit spreading colonies on agar plates which allows for easy identification of motility mutants. The bacteroidete gliding motility system is related to the Por Secretion System (PorSS) with motility genes such as gldN also being required for protein secretion[13].

Proteins secreted by PorSS are another possible source of virulence. Studies in *F. johnsoniae* indicate that gldJ is involved in the secretion of chitinase, an extra cellular enzyme which degrades the polysaccharide chitin[13]. Chondroitinase clsA is an extracellular enzyme, which may be involved in connective tissue digestion to allow bacteria to infect the host organism[18]. Whether chondroitinase is secreted by the PorSS is unknown. Chondroitinase degrades chondroitin in the extracellular matrix of fish tissue which may cause the saddleback regions present in the infected fish[17]. These lesions are used as a tool to assess virulence in the *Danio rerio* (zebra fish) model system of Columnaris disease. High virulence strains tend to kill the fish before a lesion can form while low virulence strains produce sizable legions because the fish live longer[18].

An objective of this study is to determine if gliding motility and/or digestive enzymes have a role in virulence. In 2008, Staroscik and colleagues[16] developed molecular techniques usable in certain virulent strains of *F. columnare* and generated a *gldJ*- mutant which was non-motile. Subsequent work by the same group produced a *clsA*- mutant deficient in chondroitinase activity. Both of these mutants were generated in the C#2 wild type by homologous recombination with a suicide vector containing an internal fragment of the target gene, conferring cefoxitin resistance and disrupting the gene in question. Three strains of *F. columnare* used to test virulence in the present study were: wild type (C#2), the mutant deficient in motility gene *gldJ* (*gldJ*-), and the mutant deficient in the digestive enzyme *clsA* (*clsA*-).

Because of the difficulty in producing and maintaining insertional knockouts in *F. columnare*, another objective of this study is to determine whether strains useful in generating unmarked deletion mutants retained virulence. The C#2 wild type and mutant strains (*clsA*-, *gldJ*-) were compared to the 94-081 wild type and mutant strains derived from it (Sm2, Sm3). Sm2 and Sm3 are spontaneous mutants with altered ribosomal proteins resulting in resistance to streptomycin. The 94-081 strain and its mutants were tested for virulence because these spontaneous streptomycin resistant mutants can be used to produce unmarked deletions using a system recently described for *F. johnsonaie*. A future goal is to use this system to make additional, unmarked deletion mutants in *F. columnare* for use in virulence testing[11].

2. Methods and Materials

2.1 Culture Growth

Strains of *F. columnare* were streaked onto 1/10 castone yeast CYE plates from cultures stored frozen at -80 ^{o}C and incubated at 30 ^{o}C. After 48 h, colonies were picked off plates and transferred to 5 mL of maltose (MAT) or Shieh media.[3] The C#2 wildtype and its derived mutants were grown in tubes of MAT media while the 94-081 wildtype and its derived mutants were grown in tubes of shieh media. MAT media used for clsA- and gldJ- mutants contained cefoxitin at 10 μg/mL. Shieh media used for Sm2 and Sm3 mutants contained streptomycin at 10 μg/mL, the tubes were placed in a shaking incubator at 27 ^{o}C and 150 rpm. After 24 h, 1.0 mL was transferred to a 250 mL Klett flask with 24 mL of MAT and placed in the shaker for approximately 4-5 h.

2.2 Growth Curves

Klett readings were taken every 2 h for up to 12 h. Readings were also taken at 24 h and 32 h after initial inoculation. The optical density results were plotted versus time to yield a growth curve for each strain.

2.3 Viable Counts

A sample was taken from each Klett flask between ~4-5 h after inoculation and serially diluted with sterile H_2O to a concentration 10^{-6}. The dilutions for each culture were plated on 1/10 CYE and incubated for approximately 48 h at 30 ^{o}C before counting. The number of colony-forming unit (CFU) was then recorded.

2.4 Fish Infection

Approximately 4-5 h after cultures were transferred to the Klett flask, optical density readings were taken and each strain was diluted to approximately 10^{7} and 10^{8} CFU/mL in sterile dH_2O. Diluted cultures (100 mL) were placed in 250 mL beakers[14]. A separate control beaker contained 100 mL of sterile H_2O. Zebra fish were anesthetized with MS-222 at 3.84 mg/ mL for 30-60 seconds and 2-4 scales were removed from the right side of the dorsal fin using a 28 gauge needle. Fish were then placed in the infection beakers, six in each beaker, for one hour before being transferred to separate tanks. The tanks were checked over the course of 72 h, and the number of dead fish was recorded[9].

2.5 LD50 Calculation

LD 50's of each strain were calculated using the method of Reed and Muench[10].

3. Results

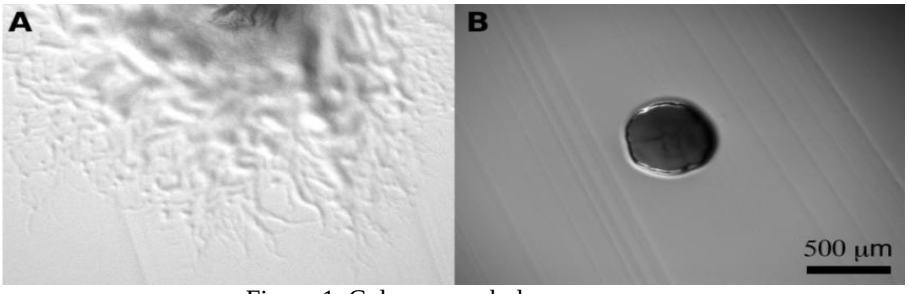
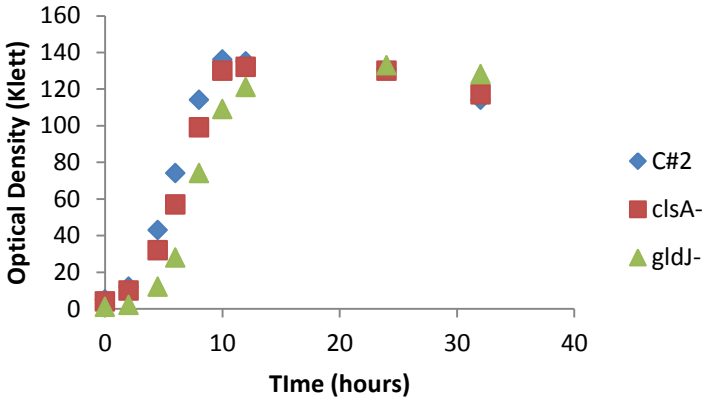


Figure 1. Colony morphology

Figure 1. Spreading (A) and non-spreading (B) colonies of *F. columnare.* The gldJ- mutant displayed non spreading growth on plates while the other *F. columnare* strains displayed spreading colonies[17].

A)



B)

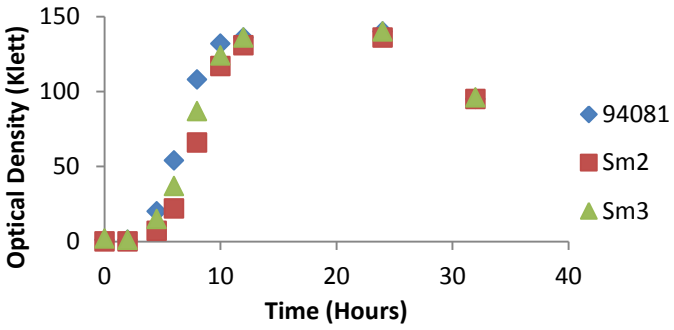


Figure 2**.** Strain Growth curves. The C#2 and clsA- strains displayed similar growth kinetics while the gldJ- strain grew at a slower rate (A). The wild type and mutant strains displayed similar growth kinetics (B).

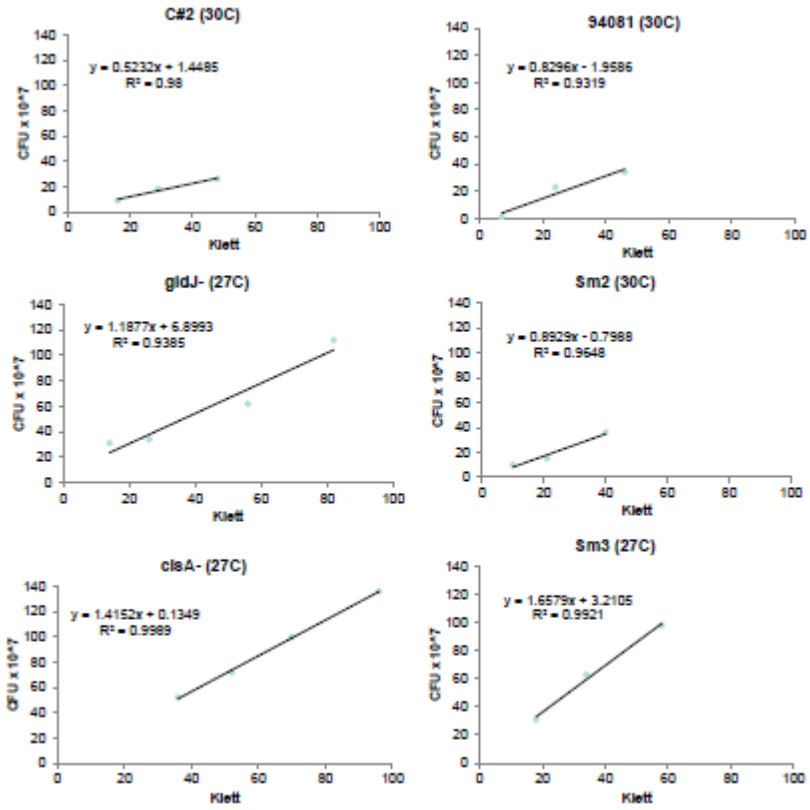


Figure 3. Viability counts and optical density comparison. The viable counts and the optical density measurements are in close agreement during early log phase.

When grown on 1/10 CYE plates all strains but the gldJ- strain displayed spreading growth (Fig. 1). The gldJ- mutant displayed non spreading growth due to the loss of a gene that has been indicated to play a role in movement.[2] The wild type C#2 strain and mutants derived from it grew planktonically in MAT media during early log phase, while the wild type 94-081 and mutants derived from it grew planktonically in Shieh media (Fig. 2). There was strong correlation between optical density and viable counts under the conditions tested, with R^2 values above 0.90 for all strains (Fig. 3). The high R^2 values allowed us to use the line equations to calculate bacterial concentrations at specific time points.

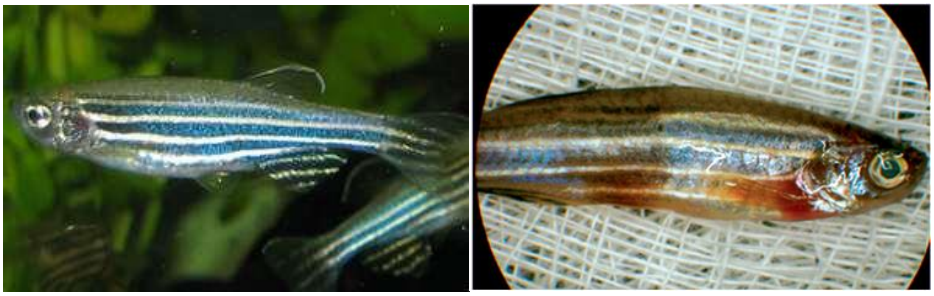


Figure 4. Healthy and infected zebra fish

Figure 4**.** Healthy zebra fish (A) and infected zebra fish with saddle back lesion (B)[9].

Table 1. The loss of the *clsA* gene decreases virulence by roughly 4 fold, while the loss of *gldJ* decreases virulence by roughly 4.5 fold. The 94081 and Sm2 strain are roughly twice as lethal as the C#2 while the Sm3 strain decreases virulence by 3 fold. The reason for lower virulence in the streptomycin resistant mutant is unknown.

Table 1. comparison of LD50 values wildtype and derived mutants

Strain	LD50 (CFU)	Fold Change
C#2	2.16×10^5	-
ClsA-	8.41×10^5	4x increase
GldJ-	9.89×10^5	4.5x increase
94081	1.29×10^5	-
Sm 2	1.84×10^5	-
Sm 3	3.83×10^5	3x increase

Fish infected with the various F. columnare strains exhibited the classic saddleback lesions (Fig. 4). LD50 calculations indicate that the C#2 wild type is more virulent in zebra fish compared to the previously published 14-56 strain.[9] Both the clsA- and gldJ- mutants were roughly 4-fold less virulent than the C#2 wild type, suggesting a role for chondroitin AC lyase and gliding motility and/or secretion in virulence. The 94-081 wild type produced a lower LD50 than C#2, indicating it is a slightly more virulent strain. The streptomycin-resistant mutants derived from 94-081 were slightly less virulent than the wild type, but the Sm2 mutant in particular was similar in virulence to the C#2 strain (Table 1).

4. Discussion

The specific causes for *F. columnare* virulence is unknown, but it has been suggested that adhesion, gliding motility, and digestive enzymes all may contribute to virulence [4,13,15]. We focused on GldJ because it is a part of the gliding motility mechanism responsible for cell movement; components of the gliding apparatus have been shown to be used for protein secretion linked to virulence in *Porphyromonas gingivalis*[13]. The chondroitinase enzyme was another focus for this study because of previous evidence that it is involved in the digestion of the mucus layer on fish[18,19].

One of the difficulties associated with assessing *F. columnare* virulence is ensuring accurate cell counts in cultures used for infection studies[7]. Multiple growth media were tested before settling on MAT media for C#2 and mutants generated from it, and Shieh media for 94-081 and mutants generated from it (data not shown). The gldJ- mutant showed a slightly slower growth rate than the C#2 wild type strain, which may be due to a nonfunctional gliding motility system. The clsA- mutant showed a similar growth rate compared to the C#2 wild type (Fig. 2A). The Sm2 and Sm3 mutants showed a similar growth rate compared to the 94-081 wild type (Fig. 2B). Both growth profiles allowed for sufficient log phase cells to be harvested for fish infection. During early log phase the optical density and viable counts were in close agreement (Fig. 3).This agreement allowed the correct number of cells to be harvested for fish infection experiments during log phase by measuring optical density. Making dilutions of cells while they were in the growth phase minimized the number of dead cells in the culture that could possibly affect the virulence of the strains and minimized cell clumping that could affect viable counts required for LD50 calculations[6].

The LD50 levels were used to determine the relative virulence of the mutant strains compared to the wild type strain (Table1). The clsA- mutant is about four times less virulent than the C#2 wild type, so we can conclude that disrupting the chondroitinase gene leads to a decrease in virulence. Based on these results, it is likely that this digestive enzyme is a contributing factor to *F. columnare* pathogenesis[19]. The gldJ- mutant was about 4.5 times less virulent than the C#2 wild type, so we could conclude that gliding motility and/or protein secretion plays a role in virulence[13]. The gldJ- mutation could lead to a decrease in virulence because the cells are unable to move[2]. It is also possible that the virulence defect seen in the gldJ- mutant is due to a loss of the ability to secrete specific proteins[8]. To separate these two possibilities, mutants will need to be generated in genes with clear homologs in the *Porphyromonas*, secretion system, such as gldN[13].

The Sm3 mutant was roughly three times less virulent than the 94-081 wildtype. The difference between the mutant strain and the wild type is a single amino acid change in the rpsL ribosomal protein which allows the mutant to be streptomycin resistant[12]. Further testing needs to be done with this mutant to determine why it is less virulent when compared to the wild type. The Sm2 mutant showed a similar virulence level to the 94-081 wild type. We can conclude that this mutant strain should be useful for generating unmarked deletion mutants to test additional factors for virulence in zebra fish model system. In future experiments, the Sm2 strain will be used to construct new mutant strains. These unmarked deletion mutants will be constructed using the deletion strategy designed by Rhodes and tested in zebra fish models[11].

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6. References Cited

1. Austin, B., and D. A. Austin**.** 2007. Bacterial Fish Pathogens: Diseases of farmed and wild fish, 4 ed. Springer, Chichester, England.
2. Braun, T. F., and M. J. McBride. 2005. Flavobacterium Johnsoniae GldJ Is a Lipoprotein That Is Required for Gliding Motility. *Journal of Bacteriology*, 187.8, 2628-2637.
3. Cain, K. D., and B. R. LaFrentz. 2007. Laboratory maintenance of *Flavobacterium psychrophilum* and *Flavobacterium columnare*, Protocols in Microbiology. Wiley Interscience.
4. Decostere, A., F. Haesebrouck, E. Van Driessche, G. Charlier, and R. Ducatelle. 1999. Characterization of the Adhesion of Flavobacterium Columnare (Flexibacter Columnaris) to Gill Tissue. *Journal of Fish Diseases*, 22.6, 465-474.
5. Kunttu, Heidi. 2010. Characterizing the Bacterial Fish Pathogen Flavobacterium Columnare, and Some Factors Affecting Its Pathogencity. *Jyvaskyla Studies in Biological and Environmental Science*. 1-81.
6. Kunttu, H.m.t., E.i. Jokinen, E.t. Valtonen, and L.-R. Sundberg. 2011. Virulent and Nonvirulent Flavobacterium Columnare Colony Morphologies: Characterization of Chondroitin AC Lyase Activity and Adhesion to Polystyrene. *Journal of Applied Microbiology*, 111, 1319-1326.
7. LaFrentz, Br, and Kd Cain. 2007. Identification of Potential Vaccine Target Antigens by Immunoproteomic Analysis of a Virulent and a Non-virulent Strain of the Fish Pathogen Flavobacterium Psychrophilum. *Diseases of Aquatic Organisms*, 74, 37-47.
8. McBride, Mark J., and Yongtao Zhu. 2013. Gliding Motility and Por Secretion System Genes Are Widespread among Members of the Phylum Bacteroidetes. *Journal of Bacteriology*, 195, 270-78.
9. Moyer, T, Hunnicutt, D. 2007 Susceptibility of zebra fish *Danio rerio* to infection by *Flavobacterium columnare* and *F. johnsoniae*. *Diseases of Aquatic Organisms* 76, 39-44.
10. Reed LJ, Muench H. 1938 A simple method of estimating fifty percent endpoints. *American Journal of Hygiene*, 27, 493–497.

11. Rhodes, R.., H. Pucker, and M.J. McBride. 2011. Development and use of a gene deletion strategy for *Flavobacterium johnsoniae* to identify the redundant gliding motility genes *remF, remG, remH, and remI*. *Journal of Bacteriology*, 193, 2418-2428.
12. Rhodes, R. G., M. N. Samarasam, A. Shrivastava, J. M. Van Baaren, S. Pochiraju, S. Bollampalli, and M. J. McBride. 2010. Flavobacterium Johnsoniae GldN and GldO Are Partially Redundant Genes Required for Gliding Motility and Surface Localization of SprB. *Journal of Bacteriology* 192.5, 1201-1211.
13. Sato, K., M. Naito, H. Yukitake, H. Hirakawa, M. Shoji, M.J. McBride, R.G. Rhodes, and K. Nakayama. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc. Natl. Acad. Sci. USA*, 107, 276-281
14. Soltani, M., B.l. Munday, and C.m. Burke. 1996. The Relative Susceptibility of Fish to Infections by Flexibacter Columnaris and Flexibacter Maritimus. *Aquaculture*, 140, 259-264.
15. Soto, E., Mauel, MJ., Karsi, A., Lawrence, ML. 2008. Genetic and virulence characterization of *Flavobacterium columnare* from channel catfish (*Ictalurus punctatus*). *Applied Microbiology*, 104, 1302-1310.
16. Suomalainen, L-R, M. Tiirola, and E. T. Valtonen. 2006. Chondroitin AC Lyase Activity Is Related to Virulence of Fish Pathogenic Flavobacterium Columnare. *Journal of Fish Diseases*, 29.12, 757-63.
17. Staroscik, A., Hunnicutt, D., Archibald, K., Nelson, D. 2008. Development of methods for the genetic manipulation of *Flavobacterium columnare. BMC Microbiology*, 8, 115-126.
18. Stringer-Roth, K. M., W. Yunghans, and L. F. Caslake. 2002. Differences in Chondroitin AC Lyase Activity of Flavobacterium Columnare Isolates. *Journal of Fish Diseases*, 25, 687-691.
19. Thomas-Jinu S, Goodwin AE. 2004. Morphological and genetic characteristics of Flavobacterium columnare isolates: correlations with virulence in fish. *Journal of Fish Diseases*, 27, 29-35.
20. Xie, H. X., P. Nie, M.X. Chang, Y. Liu, and W.J. Yao. 2005. Gene cloning and functional analysis of glycosaminoglycan-degrading enzyme chondroitin AC lyase from Flavobacterium columnare G4. *Arch. Microbiology*, 184, 49-55.