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Analysis of Unique Genes Reveals Potential Role of Essential Amino Acid Synthesis Pathway in *Flavobacterium covae* Virulence

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ABSTRACT

Flavobacterium covae is one of the four *Flavobacterium* species causing columnaris disease among warm-water fish. *Flavobacterium covae* strain 94-081 is highly virulent in channel catfish (*Ictalurus punctatus*) compared to *F. columnare* strain ATCC 49512. This study focused on analyzing the unique genes present in the genome of *F. covae* strain 94-081 compared to the genome of *F. columnare* strain ATCC 49512. Our comparative genome analysis revealed presence of 36 proteins unique to *F. covae*. Within the unique genes, nine genes (*leuA*, *leuB*, *leuC*, *leuD*, *ilvA*, *ilvB*, *ilvC*, *ilvD*, and *ilvH*) were associated with the branched-chain amino acids (BCAA) biosynthesis pathway and seven genes (*cysD*, *cysE*, *cysG*, *cysH*, *cysI*, *cysK*, and *cysN*) were involved in the cysteine biosynthesis pathway. Among the BCAA biosynthesis-related genes, *ilvC* (ketol-acid reductoisomerase) and *ilvD* (dihydroxy-acid dehydratase) showed potential interaction with catfish proteins based on the host-pathogen interaction database analysis. To investigate the functional significance of the BCAA pathway, we generated *F. covae leuD* (*Fc*Δ*leuD*) and *ilvD* (*Fc*Δ*ilvD*) mutants. The deletion of *leuD* and *ilvD* genes did not cause growth defects, but they showed reduced virulence in catfish (47.5% and 70% mortality, respectively) compared to the wild-type strain (*Fc*WT) (100% mortality). This is the first report indicating a potential role of the BCAA pathways in *F. covae* virulence. Further studies will reveal the role of the cysteine biosynthesis pathway and other unique genes on *F. covae* virulence.

Keywords
Flavobacterium covae, columnaris, *leuD*, *ilvD*, *ilvC*, BCAA, virulence

Key Messages

- Comparative genome analysis identified 36 unique proteins in *F. covae* strain 94-081, including a branched-chain amino acid (BCAA) biosynthesis pathway (9 genes) and a cysteine biosynthesis pathway (7 genes) absent in *F. columnare*.

- Deletion of two BCAA genes (*leuD* and *ilvD*) significantly reduced *F. covae* virulence in catfish (47.5% and 70% mortality vs. 100% for wild-type) without affecting bacterial growth, indicating these pathways contribute specifically to pathogenicity.
- This is the first study linking BCAA biosynthesis to *F. covae* virulence, with host-pathogen interaction analysis suggesting that IlvC and IlvD proteins may interact with catfish proteins involved in immune response and cellular signaling.

INTRODUCTION

Columnaris disease affects freshwater fish species, causing significant economic losses in aquaculture through decreased survival, increased treatment costs, and reduced yield [1]. Columnaris outbreaks in cultured channel catfish often occur during the spring and autumn and can lead to substantial mortality rates, particularly in stress and poor environmental conditions. The disease can manifest gradually as chronic infections with a progressive increase in mortalities or rapidly cause high mortalities within a few days [2,3]. It causes "saddleback" lesions and affects fins, skin, and gills [4-6].

The causative agent of columnaris disease was first isolated by Davis in 1922 and has since been found in fresh and brackish water worldwide [7-10]. The causative agent was considered *Flavobacterium columnare*, but *F. columnare* exhibited varying colony morphologies, genetic heterogeneity, and significant variation in virulence across different fish species. Genomic analyses revealed extensive genetic diversity among *F. columnare* strains, characterized by variations in average nucleotide identity (ANI), DNA-DNA hybridization, and multilocus phylogenetic analysis involving 16S rRNA and housekeeping genes [11-14]. Based on these genetic results, *F. columnare* was classified into four different genetic clusters [14,15].

It is now well-established that columnaris disease is caused by four different *Flavobacterium* species, *F. columnare, F. covae, F. davisii,* and *F. oreochromis*, which correspond to the genomovar groups I to IV [16]. *Flavobacterium columnare* isolates exhibit higher virulence towards cold-water fish species such as rainbow trout, whereas *F. covae* is known for its heightened virulence towards warm-water fish species, particularly catfish [17-23]. While genome sequencing and comparative genome analyses of *F. columnare* and *F. covae* revealed their general features [6], the specific genetic mechanisms responsible for virulence in *F. covae* are poorly understood. In this study, we determined the function-based unique genes and pathways found in the *F. covae* genome and showed the potential role of essential amino acid synthesis pathways in *F. covae* virulence in channel catfish.

MATERIALS AND METHODS

Bacterial species and growth conditions

Bacterial strains and plasmids utilized in this study are listed in Table 1. The *F. covae* strain 94-081 was cultivated using *F. columnare* growth medium (FCGM) agar or broth at a temperature of 30°C [24]. *Escherichia coli* strains were cultured on Lysogeny agar (LA) and in Lysogeny broth (LB) by incubating at 37°C in a shaker incubator at 200 rpm. When necessary, the following

antibiotics were added to the bacterial cultures: ampicillin (100 µg/ml), colistin (12.5-25 µg/ml), and cefoxitin (10 µg/ml).

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains	Description	References
Flavobacterium covae		
Strain 94-081	Wild type	[25]
*Fc*Δ*leuD*	94-081 derivative; col; Δ*leuD*	This study
*Fc*Δ*ilvD*	94-081 derivative; col; Δ*ilvD*	This study
Escherichia coli		
BW19851λ*pir*	*RP4-2 (Km::Tn7, Tc::Mu-1), DuidA3::pir+, recA1, endA1, thi-1, hsdR17, creC510*	[26]
DH5αMCR	F−*supE44 endA thi-1* λ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169 φ80lacZ*Δ*M15 mcrA* Δ(*mrr hsdRMS mcrBC*)	[27]
Plasmids		
pCP29	ColE1 ori; (pCP1 ori); Ap^r (Cf^r Em^r); *E. coli-F. psychrophilum* shuttle plasmid	[28]

Identification of Unique Genes and Pathways

To identify genes unique to *F. covae*, nucleotide files of *F. columnare* strain ATCC 49512 (accession # NC_016510.2) and *F. covae* strain 94-081 (accession # NZ_CP013992.1) were obtained from the National Center for Biotechnology Information (NCBI). Subsequently, the nucleotide sequence files were uploaded to the RAST (Rapid Annotation using Subsystem Technology) annotation pipeline (version 2.0) [29] for annotation. Default features (RAST annotation scheme: classic RAST, gene caller: RAST, FIGfam version: Release70, automatically fix errors, fix frameshifts, build metabolic model, backfill gaps, turn on debug, and disable replication: yes, verbose level: 0) were applied during the annotation process. The SEED viewer was utilized to compare the annotated genomes of *F. columnare* strain ATCC 49512 and *F. covae* strain 94-081. The SEED viewer also facilitated KEGG metabolic analysis using comparative tools [29]. Unique genes were compared against other species using the NCBI's Basic Local Alignment Search Tool, with a sequence identity cutoff of 70% and E-value threshold of 1e-5 to define uniqueness. Comparison included closely related *Flavobacterium* species.

Host Pathogen Protein-Protein Interactions

The host-pathogen interaction database (HPIDB 3.0) was employed to investigate protein-protein interactions between *F. covae* proteins and channel catfish proteins (Accession# NC_030416). The host and pathogen files were downloaded from the NCBI and uploaded to the HPIDB database using default features (search by homologous HPI, search options: for the set of host and pathogen proteins, identify homologous interactions) [30]. Cellular locations of interacting proteins were predicted by PSORTdb 4.0 [31].

In-frame Deletion of the *Flavobacterium covae leuD* and *ilvD* Genes

The nucleotide sequences of *leuD* (accession #: AWN65_RS09580) and *ilvD* (accession #: AWN65_RS09745) were obtained from the *F. covae* genome available at NCBI (accession # NZ_CP013992.1) [13]. Markerless in-frame gene deletion was conducted following the previously published procedures [32]. Briefly, primers (Table 2) were designed using online primer design software [33], and the upstream and downstream regions of the genes were amplified separately and then combined through overlap extension PCR [34]. The resulting product and the suicide plasmid pCP29 were digested with the same restriction enzymes and then ligated to the pCP29 plasmid [28]. The recombinant plasmid was isolated from *E. coli* DH5-α [35] and electroporated into *E. coli* BW19851 [26] for the conjugative transfer of pCP29 into *F. covae*.

Table 2. Primer sequences for in-frame deletion.

Primers	Sequence (5' to 3')[a]	Restriction enzyme
*leuD*EF01	**atgtcgac**TTGGCACAAGTCAGGTAGCAC	SalI
*leuD*IR01	ACGAGCAGGTATAATTTGGTCTG	
*leuD*IF01	cagaccaaattatacctgctcgtGAAGCCTTTGAAAAATCAAGAAA	
*leuD*ER01	**atctgcag**TCCAACAACTTCCGTTGAATAA	PstI
*ilvD*EF01	**atgtcgac**AACATCGAACCATACATTCGTG	SalI
*ilvD*IR01	TTGGGTAATCGTTTTGCTGT	
*ilvD*IF01	acagcaaaacgattacccaaGCCTCTCAAGGATGTGTTACCG	
*ilvD*ER01	**atctgcag**TCTATCATCAAACCGCATTCC	PstI

[a] Lowercase bold letters show restriction enzyme recognition sequences added to primers. Lowercase letters in IF01 primers indicate reverse complemented IR01 reverse primer sequences.

Transformants were selected on FCGM agar supplemented with cefoxitin and colistin. Confirmation of the merodiploid first recombinant event was achieved through colony PCR. PCR amplicons were examined by agarose gel electrophoresis to verify expected product size and primer specificity, followed by amplicon sequencing for confirmation. Confirmed positive colonies were streaked onto FCGM agar supplemented with colistin to screen for the second recombinant event and loss of the wild-type gene. Subsequently, these colonies were re-streaked on FCGM agar plates containing colistin and FCGM agar plates containing cefoxitin. Positive colonies that grew on FCGM agar with colistin but not on FCGM agar with cefoxitin were selected, and PCR was performed to confirm the gene deletion and absence of the wild-type gene. For validation of the *F. covae* mutants, the PCR product was sequenced. The new strains were designated as *Fc*Δ*leuD* and *Fc*Δ*ilvD*.

Bacterial Growth Kinetics

Overnight cultures of *F. covae* mutants (*Fc*Δ*leuD* and *Fc*Δ*ilvD*) and wild-type (*Fc*WT) were prepared, and the optical densities at 600 nm (OD_{600}) were measured. After density adjustment, cultures were diluted 1:100 with fresh FCGM and cultivated at 30°C. The OD_{600} values were measured every hour for a duration of 72 hours. The average optical absorbance at each time point was calculated and used to determine the bacterial growth rate. Growth curves were

statistically compared using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$.

Virulence of Mutants in Catfish

Experimental infections of catfish were conducted under an approved protocol of the Mississippi State University Institutional Animal Care and Use Committee following the methodology previously described [6]. Specifically, specific pathogen-free (SPF) catfish were randomly assigned to 40-liter tanks (10 fish per tank), each treatment consisting of 4 replicates. Personnel recording clinical signs and mortalities were blinded to treatment allocation during the observation period. Catfish fingerlings had an average weight of 14.2±2.9 grams and a length of 22.1±0.6 centimeters. Tanks were supplied with flow-through dechlorinated municipal water, and water temperature was maintained at 30°C throughout the experiments. For experimental infections, *FcΔleuD*, *FcΔilvD*, or *FcWT* cultures were grown in a shaking incubator at 150 rpm for 18 h. After adjustment of OD_{600} to 0.6, 50 ml cultures were added to assigned tanks with 10 liters of water (~5 x 10^6 CFU/ml of water). The exposure lasted 1 hour, after which the water flow was restored. A sham control treatment (sterile FCGM broth) was also included. Clinical signs of the disease were monitored, and fish mortalities were recorded daily for 8 days, and the percent and cumulative mortalities were calculated for each experimental group. Kaplan-Meier survival analysis was performed to compare the mortality rates of treatment and control groups. Pairwise comparisons were conducted using the Mantel-Cox Log Rank test (IBM SPSS v 29 Armonk, NY). The significance threshold was $p < 0.05$.

RESULTS

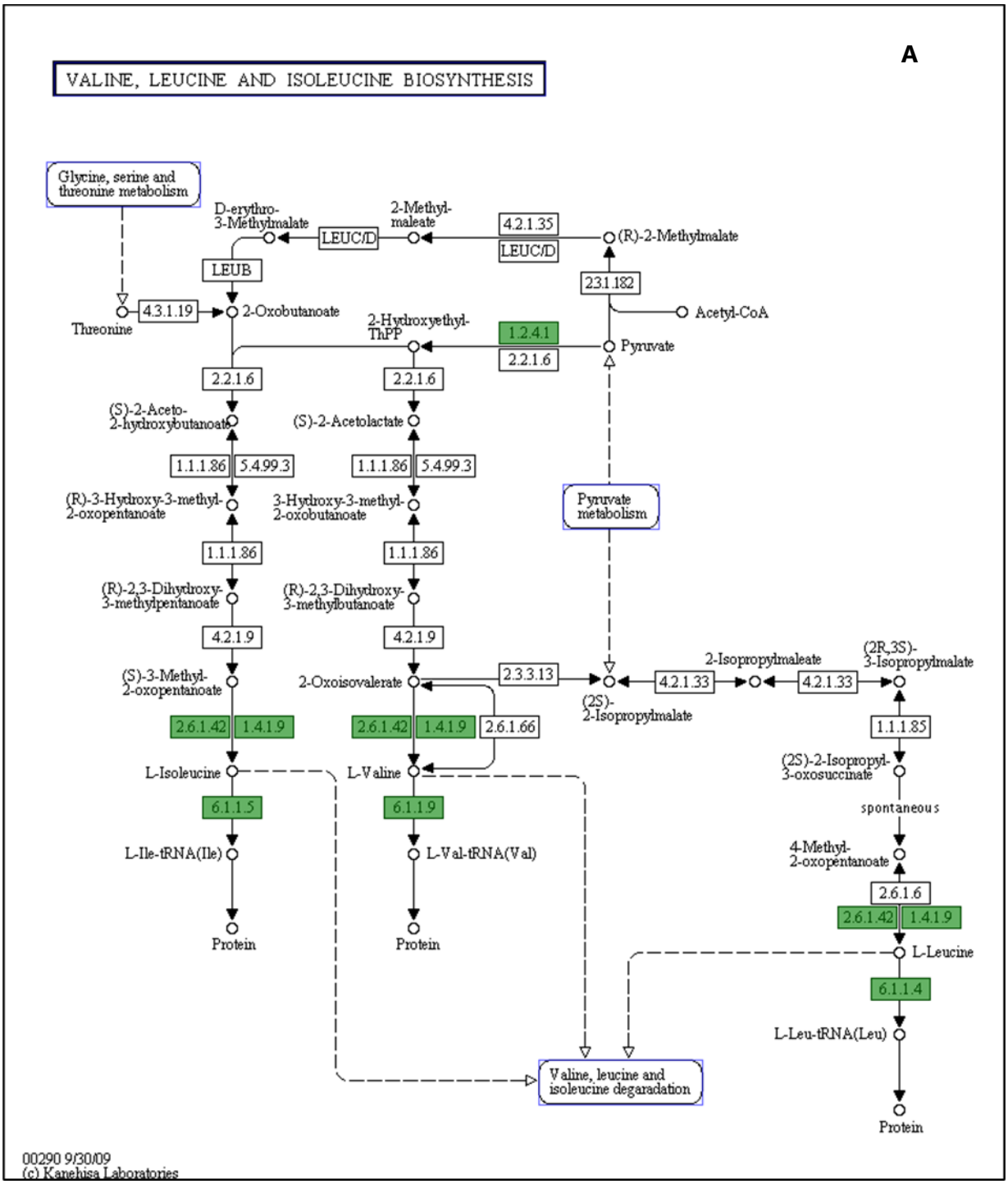
Unique Proteins and Pathways of *Flavobacterium covae*

We identified 36 unique protein-coding genes in *F. covae* strain 94-081 compared to *F. columnare* strain ATCC 49512 based on gene annotations. These unique proteins were categorized based on their roles (Table 3). Through pathway analysis, we identified that the branched-chain amino acid (BCAA) biosynthesis pathway was present in *F. covae*, *F. davisii*, and *F. oreochromis*, while not in *F. columnare*. This pathway comprises two clusters of 9 unique genes (*leuA*, *leuB*, *leuC*, *leuD*) and (*ilvA*, *ilvB*, *ilvC*, *ilvD*, and *ilvH*). Similarly, the cysteine biosynthesis pathway was present only in *F. covae* and *F. davisii*. The valine-leucine-isoleucine biosynthesis pathway of *F. columnare* and *F. covae* are depicted in Figure 1A and 1B, respectively.

Table 3. *F. covae* 94-081 unique protein-coding genes

Protein_ID	*Gene*	RAST Annotation Subsystem	NCBI annotation
WP_060382961.1	*leuA*	Branched-Chain Amino Acid Biosynthesis	2-isopropylmalate synthase
WP_060382962.1	*leuC*	Branched-Chain Amino Acid Biosynthesis	3-isopropylmalate dehydratase large subunit
WP_060382963.1	*leuD*	Branched-Chain Amino Acid Biosynthesis	3-isopropylmalate dehydratase small subunit
WP_060382964.1	*leuB*	Branched-Chain Amino Acid Biosynthesis	3-isopropylmalate dehydrogenase

WP_060382987.1	*ilvA*	Branched-Chain Amino Acid Biosynthesis	Threonine dehydratase
WP_060382988.1	*ilvC*	Branched-Chain Amino Acid Biosynthesis	Ketol-acid reductoisomerase
WP_081078427.1	*ilvH*	Acetolactate synthase subunits	Acetolactate synthase small subunit
WP_060382990.1	*ilvB*	Acetolactate synthase subunits	Biosynthetic-type acetolactate synthase large subunit
WP_060382991.1	*ilvD*	Branched-Chain Amino Acid Biosynthesis	Dihydroxy-acid dehydratase
WP_060383305.1	*cysI*	Cysteine Biosynthesis	NADPH-dependent assimilatory sulfite reductase hemoprotein subunit
WP_060383307.1	*cysG*	Heme and Siroheme Biosynthesis	Uroporphyrinogen-III C-methyltransferase
WP_060383308.1	*cysK*	Cysteine Biosynthesis	Cysteine synthase A
WP_060383309.1	*cysE*	Cysteine Biosynthesis	Serine acetyltransferase
WP_060383310.1	*cysN*	Cysteine Biosynthesis	GTP-binding protein
WP_060383311.1	*cysD*	Cysteine Biosynthesis	Sulfate adenylyltransferase subunit 2
WP_060383312.1	*cysH*	Cysteine Biosynthesis	Phosphoadenylyl-sulfate reductase
WP_060382296.1		Phage tail fiber proteins	Hypothetical protein
WP_060382475.1		N-linked Glycosylation in Bacteria	Glycosyltransferase
WP_060382615.1		ABC transporter oligopeptide (TC 3.A.1.5.1)	Hypothetical protein
WP_060383861.1		Ton and Tol transport systems	TonB-dependent receptor
WP_060382696.1		Biogenesis of c-type cytochromes	TlpA family protein disulfide reductase
WP_060381414.1		cAMP signaling in bacteria	Clp protease ClpP
WP_060382767.1		CBSS-84588.1.peg.1247	Serine hydrolase
WP_060382866.1	*speG*	Arginine Biosynthesis -- gjo	N-acetyltransferase
WP_060383088.1		Rhamnose containing glycans	ABC transporter ATP-binding protein
WP_060383884.1	*epsM*	N-linked Glycosylation in Bacteria	Acetyltransferase
WP_060383106.1		CBSS-296591.1.peg.2330	Sugar transferase
WP_060383107.1		CBSS-296591.1.peg.2330	Glycosyltransferase family 1 protein
WP_060383303.1		Heme and Siroheme Biosynthesis	Siroheme synthase
WP_060383507.1	*mazF*	Phd-Doc, YdcE-YdcD toxin-antitoxin systems	Type II toxin-antitoxin system PemK/MazF family toxin
WP_060383508.1	*mazE*	Phd-Doc, YdcE-YdcD toxin-antitoxin systems	AbrB/MazE/SpoVT family DNA-binding domain-containing protein
WP_060381524.1	*cas2*	CRISPRs	CRISPR-associated endonuclease Cas2
WP_060381594.1		ABC transporter oligopeptide (TC 3.A.1.5.1)	TonB-dependent receptor
WP_060381731.1	*rnz*	Beta-lactamase	Peptidase
WP_060381334.1	*wcaJ*	CBSS-296591.1.peg.2330	Hypothetical protein
WP_060382054.1		Group II intron-associated genes	RNA-directed DNA polymerase



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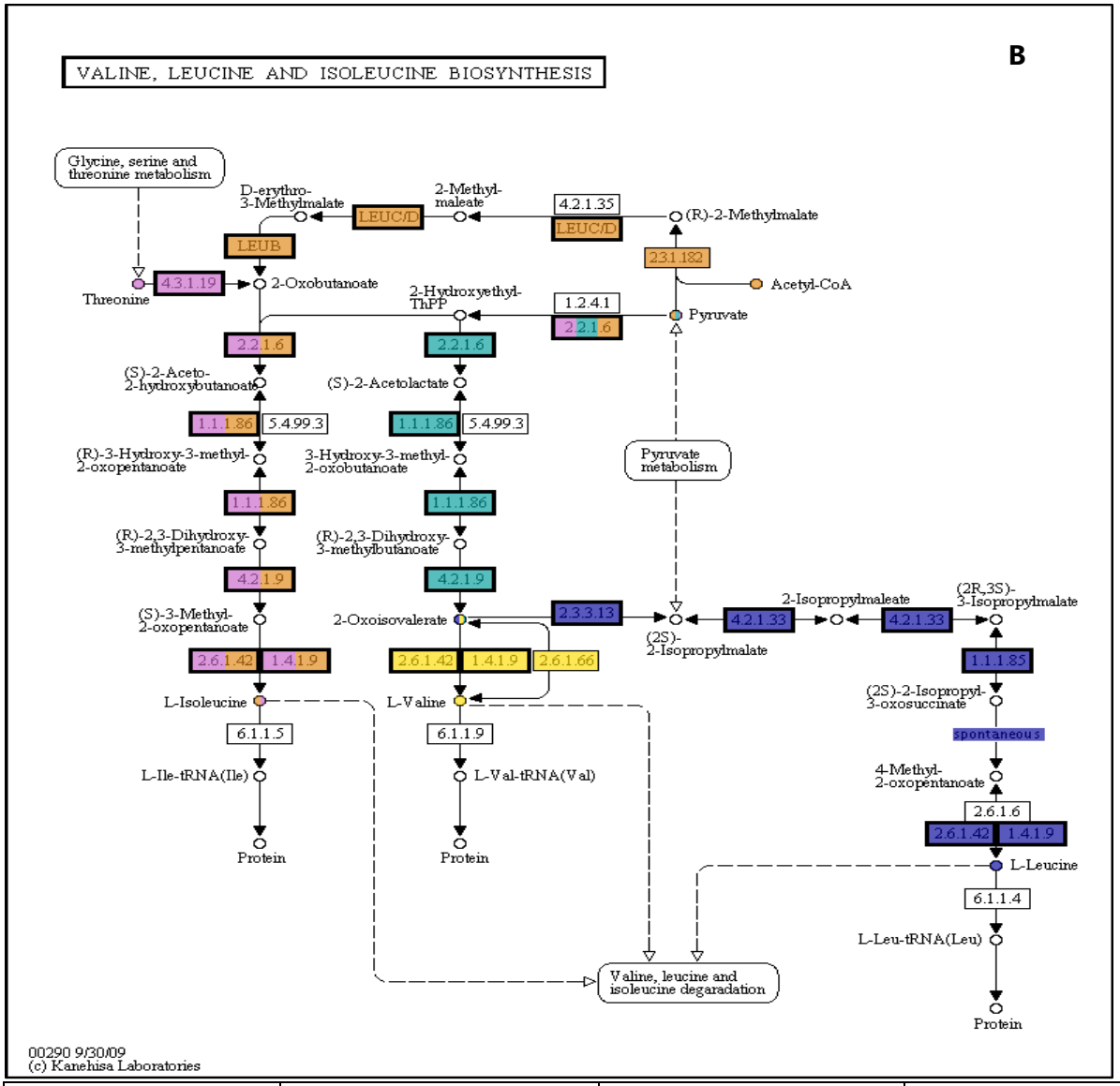

Scenario	Input Compounds	Output Compounds	Status in *F. covae*
AcetylCoA and Pyruvate to Isoleucine	Acetyl-CoA Pyruvate	L-Isoleucine	incomplete
Oxoisovalerate to Leucine	3-Methyl-2-oxobutanoic acid	L-Leucine	complete
Oxoisovalerate to Valine	3-Methyl-2-oxobutanoic acid	L-Valine	complete
Pyruvate to Oxoisovalerate	Pyruvate	3-Methyl-2-oxobutanoic acid	complete
Threonine and Pyruvate to Isoleucine	L-Threonine Pyruvate	L-Isoleucine	complete

Figure 1. (A) *Flavobacterium columnare* valine-leucine-isoleucine biosynthesis pathway. The green color shows the genes present in *F. columnare*. (B) *Flavobacterium covae* valine-leucine-isoleucine biosynthesis pathway. The colors show the genes in *F. covae*, with different colors representing pathways within the valine-leucine-isoleucine biosynthesis pathway.

Host-Pathogen Protein-Protein Interactions

The host-pathogen protein-protein interaction analysis revealed that 2 of the 36 unique proteins putatively interact with catfish proteins. Specifically, the *ilvC* gene was found to potentially interact with 2 catfish proteins, while the *ilvD* gene showed predicted interactions with 7 catfish proteins. The details of the host and pathogen proteins involved in these interactions are presented in Table 4. The predicted locations of IlvC and IlvD proteins were cytoplasmic.

Table 4. Unique *F. covae* proteins with potential interaction with channel catfish proteins.

Gene	Protein	Protein ID	Host Protein ID	Host Protein
ilvC	ketol-acid reductoisomerase	WP_060382988.1	XP_017337580.1	Chromodomain-helicase-DNA-binding protein 4
			XP_017337579.1	Chromodomain-helicase-DNA-binding protein 4
ilvD	dihydroxy-acid dehydratase	WP_060382991.1	XP_017311531.1	Exocyst complex component 1 isoform X1
			XP_017311532.1	Exocyst complex component 1 isoform X2
			XP_017342785.1	Nascent polypeptide-associated complex subunit alpha isoform X3
			XP_017342786.1	Nascent polypeptide-associated complex subunit alpha isoform X3
			XP_017342787.1	Nascent polypeptide-associated complex subunit alpha isoform X4
			XP_017342788.1	Nascent polypeptide-associated complex subunit alpha isoform X4
			XP_017343604.1	Ras-related C3 botulinum toxin substrate 2

Flavobacterium covae leuD and *ilvD* Mutants

We successfully performed in-frame deletions of *leuD* and *ilvD* in *F. covae*. In *Fc*Δ*leuD*, 78% of the *leuD* gene was deleted, while 96% of the *ilvD* gene was eliminated in *Fc*Δ*ilvD* (Table 5).

Table 5. Deleted *F. covae* proteins and deleted gene properties.

Protein	Gene	GL	DR	RR	DP
3-isopropylmalate dehydratase small subunit	*leuD*	613	477	136	77.8%
Dihydroxy-acid dehydratase	*ilvD*	1674	1611	63	96.2%

Abbreviations: GL: Gene length, DR: deleted region, RR: Remaining region, DP: Deletion percentage.

Bacterial Growth Kinetics

The growth kinetics of *Fc*Δ*leuD*, *Fc*Δ*ilvD*, and *Fc*WT exhibited a similar pattern with no statistically significant differences ($p > 0.05$), and cultures reached the plateau stage around 44 h (Figure 2).

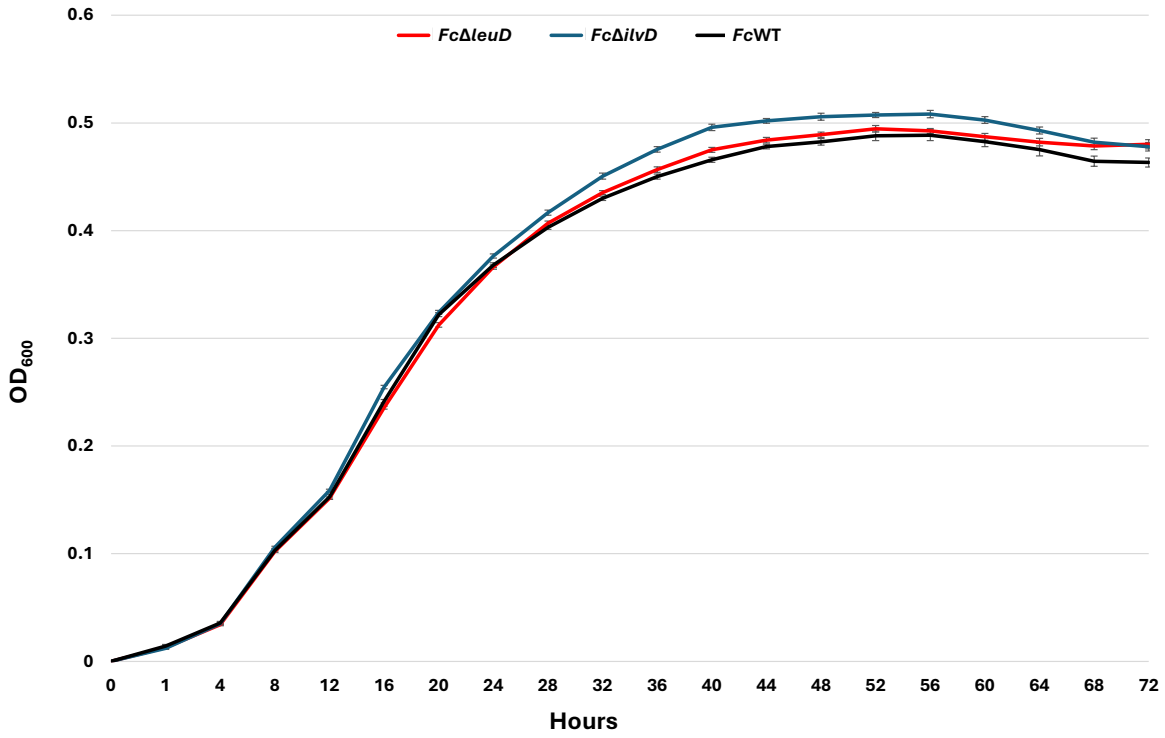

Figure 2. Growth kinetics of mutants and wild-type *F. covae*.

Virulence in Catfish

There was a significant difference in fish mortalities among all treatment groups ($p < 0.05$) (Figure 3). Mutant strains *Fc*Δ*leuD* and *Fc*Δ*ilvD* caused 47.5% and 70% mortality in catfish, respectively, while the mortality rate of *Fc*WT was 100% (Figure 3A). The progression of mortalities varied among the challenge groups. Mortalities in the *Fc*Δ*leuD* treatment started to manifest after two days. In contrast, mortalities in *Fc*WT and *Fc*Δ*ilvD* were observed as early as one day after the challenge. Peak mortalities in all treatments occurred on the second day (Figure 3B).

DISCUSSION

In this study, our primary objective was to study unique genes of *F. covae* strain 94-081 (pathogenic to catfish) compared to *F. columnare* strain ATCC 49512 (not pathogenic in catfish). Although features of *F. covae* were previously reported [6], a detailed analysis of the unique pathways has not been performed. The pathway analysis revealed seven unique proteins involved in the cysteine biosynthesis pathway, and they were present only in *F. covae* and *F. davisii*. This pathway may contribute to resistance to oxidative stress or environmental survival. Cysteine serves as a precursor for the primary path of organic sulfur combination into cellular components and methionine biosynthesis [36,37]. The cysteine biosynthesis pathway is crucial for bacterial growth and function, detoxification, and metabolic processes. Even though this pathway is present only in *F. covae* and *F. davisii*, alternative pathways in *F. columnare* and *F. oreochromis* may be present, and hence, further studies are needed to understand the role of cysteine biosynthesis pathway in *F. covae* virulence.

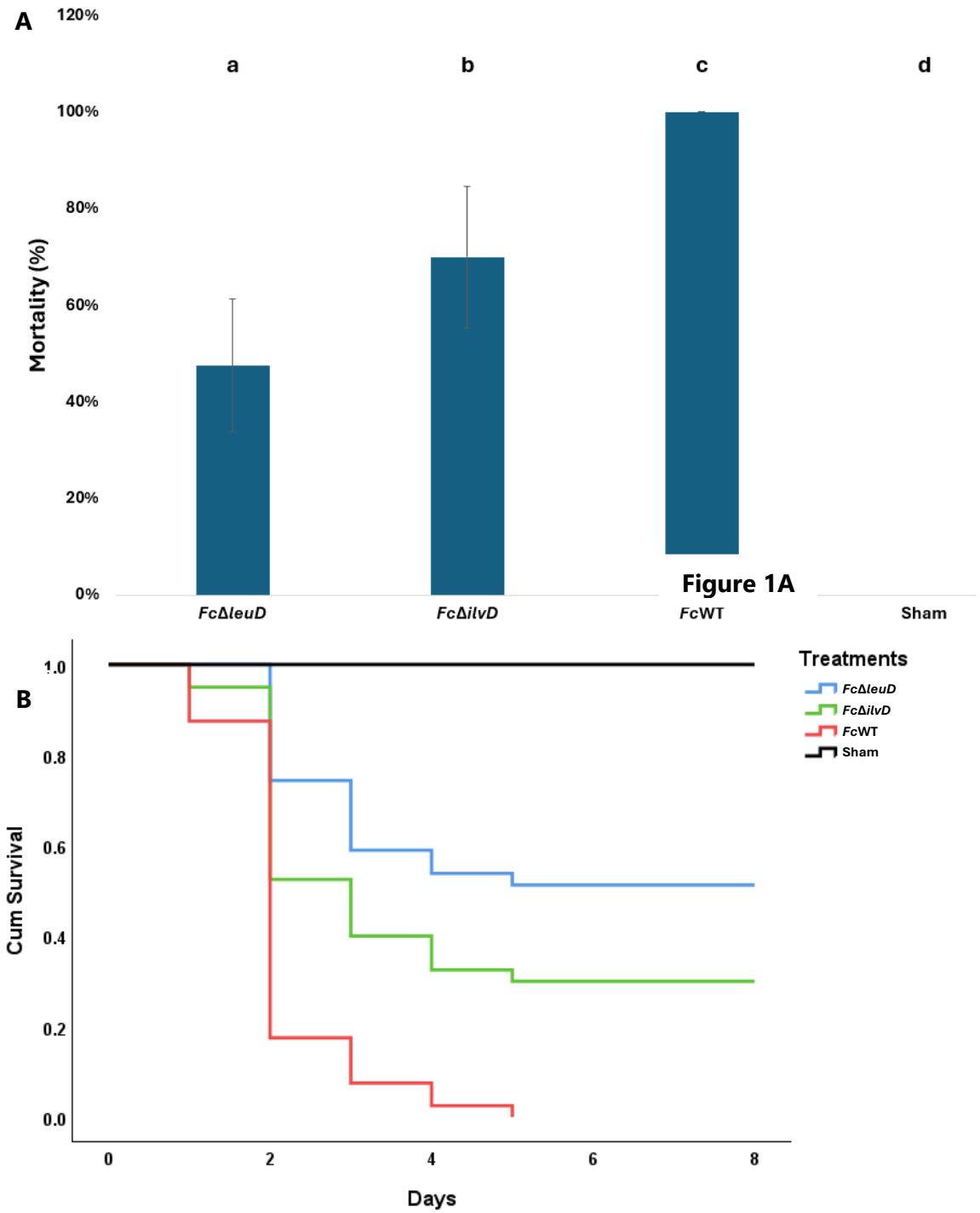

Figure 3. (A) Endpoint mortality and (B) cumulative survival among treatment groups. The small letters above bars indicate statistical significance at $p < 0.05$.

The branched-chain amino acid (BCAA) biosynthesis pathway was present in *F. covae*, *F. davisii*, and *F. oreochromis,* while not in *F. columnare*. The *F. covae* BCAA pathways included nine genes (*leuA*, *leuB*, *leuC*, *leuD*, *ilvA*, *ilvB*, *ilvC*, *ilvD*, *and ilvH*). The branched-chain amino acids are synthesized in fungi, plants, and bacteria but not in animals and regulate many biological

activities, such as growth and protein synthesis [38,39]. *Listeria monocytogenes* responds to BCAA deficiency through increasing virulence gene expression in mammalian cells [40]. The BCAA transporters affect *Bacillus anthracis* virulence and growth [38]. Interestingly, the expression of genes encoding the BCAA biosynthesis pathway in *Bacillus cereus* is predominantly enriched by a quorum-sensing effector called peptide-activating PlcR (PapR) [41]. In *Bacillus cereus*, PapR plays a crucial role as a regulator of virulence factors, and it is involved in communication, adaptation, and survival in various environments [41-43].

In catfish, both *leuD* and *ilvD* mutants exhibited attenuation, possibly due to the adverse effects of these mutations on bacterial adaptation within the host. However, the underlying mechanisms are not yet fully understood. The *leuD* gene is a component of the PhoPR two-component system and plays a crucial role in the oxidative stress response. It is also an essential enzyme involved in the valine-leucine-isoleucine biosynthesis pathway in *Mycobacterium tuberculosis*. Studies have shown that the *leuD* mutant exhibits enhanced protective immunity against *Mycobacterium avium subsp. paratuberculosis* in mouse models, making it a potential attenuated vaccine candidate [44-46]. A *leuD* mutant strain of *M. avium subsp. paratuberculosis* lost the capability of utilizing several essential nutrients such as nitrogen, carbon, phosphorus, sulfur, and other nutrient supplements as energy sources. Under different stress conditions, the *leuD* mutant strain exhibits diverse expression patterns in over 100 genes. Furthermore, the mutant strain displays a 30% reduction in fatty acid content, indicating an impact on the metabolic pathway involving fatty acids [47]. These findings highlight the multifaceted role of *leuD* in pathogenicity and metabolic adaptation in various bacterial species.

The *ilvD* plays a crucial role in the valine-leucine-isoleucine biosynthesis pathway. The difference in virulence observed between *Fc*Δ*leuD* and *Fc*Δ*ilvD* mutants suggests that isoleucine availability or host-compensated uptake may explain the partially retained virulence of the *Fc*Δ*ilvD* mutant. This pathway exhibits active involvement in the survival of *M. tuberculosis* under both normal and stress conditions [48]. Furthermore, *ilvD* is essential for the virulence of *Aspergillus fumigatus* in murine infection models [49]. As one of the essential enzymes in branched-chain amino acid (BCAA) biosynthesis, *ilvD* also plays a significant role; supplementation with BCAAs promotes the aerobic growth of *Salmonella typhimurium* [50]. In *Bacillus anthracis*, *ilvD* has a crucial role in isoleucine production in a nutrient-restrictive environment like its mammalian host [51].

Understanding host-pathogen interactions is crucial for identifying potential targets and developing effective disease control strategies [30]. In our study, we made an intriguing discovery regarding the predicted interaction between unique *F. covae* proteins and catfish proteins. The IlvC, which is the second enzyme in the BCAA biosynthesis, regulates many functional activities in organisms [39]. It interacts with channel catfish chromodomain helicase DNA-binding protein 4 that regulates gene expression and DNA-damage responses [52]. The IlvD interacts with the exocyst complex component 1 (EXOC1), a protein-coding gene essential for targeting exocytic vesicles to specific docking sites on the plasma membrane [53]. The IlvD also interacts with the nascent polypeptide-associated complex subunit alpha (NACα) that is involved in the innate immune response to pathogens such as *V. anguillarum* and *E. tarda* in crab and fish [54-56]. Moreover, IlvD interacts with ras-related C3 botulinum toxin substrate 2 (Rac2), which is a

member of the Rho family of GTPases [57]. It regulates various cellular activities, such as controlling cell growth and cytoskeletal reorganization [58]. Amino acids are essential for protein synthesis, metabolic physiology, and signaling in all organisms, but animals cannot synthesize several of them. Microbial-synthesized essential amino acids may affect health and disease by changing metabolic pathways in their hosts [59]. Specifically, two unique proteins, IlvC and IlvD, were predicted to be located in the cytoplasm and interact with catfish proteins.

In summary, although the lack of protein-protein interaction validation limits our study, these findings contribute to our understanding of the molecular mechanisms underlying the pathogenicity of *F. covae* in catfish and highlight potential targets for future studies aimed at disease prevention and control strategies.

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AUTHOR CONTRIBUTIONS

AK and ML performed the conceptualization, supervised the project, provided the resources, and writing. SK, SWN, and HA performed the experiments, data analysis, and writing.

DATA AND CODE AVAILABILITY

All data presented in this study are included in the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Mississippi State University (Protocol Number: 15-043). All efforts were made to minimize animal suffering.

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