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Post-transcriptional regulation through alternative splicing after infection with *Flavobacterium columnare* in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

Columnaris disease has long been recognized as a serious problem worldwide which affects both wild and cultured freshwater fish including the commercially important channel catfish (*Ictalurus punctatus*). The fundamental molecular mechanisms of the host immune response to the causative agent *Flavobacterium columnare* remain unclear, though gene expression analysis after the bacterial infection has been conducted. Alternative splicing, a post-transcriptional regulation process to modulate gene expression and increase the proteomic diversity, has not yet been studied in channel catfish following infection with *F. columnare*. In this study, genomic information and RNA-Seq datasets of channel catfish were used to characterize the changes of alternative splicing after the infection. Alternative splicing was shown to be induced by *F. columnare* infection, with 8.0% increase in alternative splicing event at early infection stage. Intriguingly, genes involved in RNA binding and RNA splicing themselves were significantly enriched in differentially alternatively spliced (DAS) gene sets after infection. This finding was consistent with our previous study in channel catfish following infection with *Edwardsiella ictaluri*. It was suggested to be a universal mechanism that genes involved in RNA binding and splicing were regulated to undergo differential alternative splicing after stresses in channel catfish. Moreover, many immune genes were observed to be differentially alternatively spliced after infection. Further studies need to be performed to get a deeper view of molecular regulation on alternative splicing after stresses, setting a foundation for developing catfish broodstocks with enhanced disease resistance.

1. Introduction

Columnaris disease, caused by *Flavobacterium columnare*, is the most frequently occurring disease in the aquaculture industries around the world. First described by Davis in 1922 [1], the disease was named as columnaris because the causal bacterial cells in a wet mount preparation of affected gills and fins were arranged in columnar aggregations. The pathogen *F. columnare* is a Gram-negative, rod-shaped bacterium that causes symptoms such as saddleback lesions, fin erosion, and gill necrosis [2,3]. It is pathogenic to a wide range of freshwater fish species, including zebrafish (*Danio rerio*) [4,5], striped bass (*Morone saxatilis*) [6], perch (*Perca fluviatilis*) [7], common carp (*Cyprinus carpio*) [8], Nile tilapia (*Oreochromis niloticus*) [9], rainbow trout (*Oncorhynchus mykiss*) [10,11], and channel catfish (*Ictalurus punctatus*) [4,5,12]. The channel catfish aquaculture industry in the United States has been severely impacted by columnaris disease, causing economic loss of approximately $30 million annually [13]. In pond aquaculture system, mortality rates of adults and fingerlings can reach 60% and 90%, respectively [14].

The pathogenesis of columnaris disease has been extensively studied, while little is still known about the molecular mechanisms underlying the disease [15]. The adhesion ability, especially to gill tissues, was determined to be a prerequisite for the successful colonization and infection of *F. columnare* [4,16,17]. In channel catfish, transcriptional analyses on gene expression after *F. columnare* infection were conducted in gill tissues, and expression levels of lectins and NF-κB signaling related genes were differentially regulated [18,19]. Genome-wide association study (GWAS) has been performed in backcross hybrid catfish, and genes involved in phosphoinositide 3-kinase pathway were identified to be significantly associated with columnaris disease resistance [20]. In spite of extensive studies at the genomic and transcriptomic levels, post-transcriptional regulation of the host responses to this

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disease has not yet been known.

Alternative splicing is a prominent post-transcriptional regulatory mechanism for gene regulation in eukaryotes. It enhances the mRNA complexity by allowing the generation of multiple transcript variants from a single gene. The complexity may lead to altered mRNA localization, stability, translation or decay, as well as proteomic diversity with different structures and functions [21,22]. Alternative splicing is an extremely common regulatory mechanism [23]. For instance, nearly all multi-exon genes in human were estimated to undergo alternative splicing using high-throughput sequencing [24,25]. The inclusion of different exons spliced in mature mRNA is controlled by both specific *cis*-acting sequence elements and *trans*-acting regulatory proteins [26]. The differential use of splice sites results in complex splicing events [27] and five basic types of alternative splicing events: 1) exon skipping, 2) alternative 5′ splice site, 3) alternative 3' splice site, 4) mutually exclusive exon, and 5) intron retention.

Alternative splicing is a regulated process involved in many physiological processes, including defense responses [28–30]. It enhances the level of diversity and flexible adaptation required for a functional immune system [28]. One of the best examples is that alternative splicing facilitated the generation of diverse antigen receptors of lymphocytes that can recognize a wide range of antigens [31]. Alternative splicing has been associated with disease resistance/susceptibility in various organisms ranging from plants to mammals [32–39]. For instance, alternative splicing of *Nmnat* was reported to enhance neuroprotection under stress in *Drosophila* [33]; after infection of the mastitis virus, a novel spliced isoform of the complement component 4 gene was increased in dairy cattle [36]. Alternative splicing is regarded as an effective strategy for the regulation of immune responses against pathogen infection in mammals and teleost fish [34].

Alternative splicing was found to be important for host responses to stresses including heat stress [40] and enteric septicemia of catfish (ESC) [41] in catfish. In an effort of understanding the involvement of post-transcriptional regulation through alternative splicing in catfish after stresses, in this study, we used RNA-Seq datasets to determine the alternative splicing profiles and functional associations after *F. columnare* infection in channel catfish.

2. Materials and methods

2.1. Data collection

The channel catfish reference genome sequence was downloaded from NCBI [42]. Alternative splicing analyses were conducted using existing RNA-Seq datasets from our previous study of channel catfish in response to *F. columnare* infection [18]. In the experiment, one-year-old channel catfish (48.6 ± 2.5 g) were immersion-challenged for 2 h in 30 L aquaria with a bacterial concentration of 3×10^6 CFU/mL. The mortality began around 24 h and became widespread by 36 h following infection, and 90% mortality was observed by day 7. Gill tissues were collected at 4 h, 24 h, and 48 h time points after exposure. At each time point, 18 fish from both control groups and treatment groups were randomly selected and divided into 3 replicates (6 fish each), respectively. Total RNA was extracted from each replicate. For treatment samples, equal amount of RNA from the three treatment replicates were pooled for each time point to construct RNA-seq libraries. For control samples, a RNA pool comprised of samples of all three time points was formed together.

2.2. Identification of alternative splicing after infection

Alternative splicing profiles and their associated responding genes following infection were determined by using the bioinformatic pipeline as described in Tan et al. [41]. In brief, the paired-end raw data underwent quality control process using FASTX toolkit [43] and Trimmomatic [44]. Then, high-quality reads were aligned against the channel catfish reference genome sequence with TopHat2 [45], and the mapped reads were assembled to transcripts using Cufflinks 2.2.1 [46]. ASTALAVISTA was utilized to identify and classify alternative splicing events including five basic types and complex types [47]. Since the number of alternatively spliced genes and the number of alternative splicing events were correlated with sequencing depth [48], the same number of high-quality reads was randomly selected for the subsequent identification and comparison of alternative splicing profiles between control and infected samples.

2.3. Identification of differential alternative splicing (DAS)

The software rMATS [49] was used to define DAS by computing and comparing the inclusion level [percent spliced in (PSI)] of certain alternative splicing event between two sets of RNA-Seq datasets. Taking the example of exon skipping, PSI is referred as the ratio of junction and exon reads for the skipped exon to junction and exon reads for the exon and its two flanking exons [49,50]. An FDR-adjusted *P* value less than 0.05 was used as criteria for DAS. DAS genes (genes with differential alternative splicing events) were also determined.

2.4. Functional enrichment analysis

To reveal enriched functional classes in the DAS gene sets, a statistical overrepresentation test [51] was performed using the PANTHER classification system (http://pantherdb.org). To use PANTHER, zebrafish orthologs were searched through BLAST program and ENSEMBL database (http://www.ensembl.org).

3. Results

3.1. Alternative splicing profiles after F. columnare infection

Summary of RNA-Seq datasets used in this study is shown in Table 1. A total of over 203 million raw reads were used. After quality control and trimming, over 184 million reads were used for further analysis. These RNA-Seq reads were from gill tissues at 4 h, 24 h, and 48 h after infection with *F. columnare*.

The detailed distribution of alternative splicing events at each time point following *F. columnare* infection is summarized in Table 2 and shown in Fig. 1. Although there were variations, most alternative splicing events including exon skipping, mutually exclusive exons, alternative 5′ splice sites, and alternative 3' splice sites, were increased early at 4 h after infection. The number of alternatively spliced genes and alternative splicing events both increased quickly at early time point after infection with 8.0% and 4.4% rise, respectively. As the

Table 1
Summary of RNA-Seq datasets from paired-end reads from gill tissues of channel catfish after infection with *Flavobacterium columnare*.

Sample-type	Run	Number of raw reads	Avg.length of raw reads (bp)	Number of reads after trimming	Percentage retained
Control	SRR493667	54,327,678	101	49,251,116	90.66%
4 h	SRR493669	50,127,430	101	45,641,346	91.05%
24 h	SRR493670	48,926,940	101	44,491,540	90.93%
48 h	SRR493671	49,782,122	101	45,241,030	90.88%
Total	–	203,164,170		184,625,032	90.87%

Table 2
Alternative splicing events determined from RNA-Seq datasets of gill tissues of channel catfish after infection with *Flavobacterium columnare*.

	Control	4 h	24 h	48 h
Exon skipping	1640	1678	1466	1435
Mutually exclusive exon	87	96	87	79
Alternative 5′ splice site	652	737	754	655
Alternative 3′ splice site	1578	1644	1651	1611
Intron retention	515	685	624	713
Complex	430	456	467	390
Total	4902	5296	5049	4883

disease progressed, the initial infection-induced increase of alternative splicing returned almost to the level of the control (Fig. 1B). Detailed information of the distribution of alternative splicing events and involved genes during the infection is listed in Table S1.

3.2. Differential alternative splicing after infection

Differentially alternatively spliced (DAS) genes and events were determined by comparing those after infection with the control. A total of 4, 74, and 67 DAS events were identified at 4 h, 24 h, and 48 h after infection with *F. columnare* (Table 3), corresponding to 4, 65, and 60 DAS genes (Fig. 2), respectively. Detailed information is provided in Table S1. Many of these DAS genes were immune-related, which were presented in Table 4.

3.3. Functional enrichment analysis

Zebrafish orthologs of catfish DAS genes were searched (Table S2) and used for the PANTHER overrepresentation test to determine the enriched functional categories. From the list of 4, 65, and 60 DAS genes at 4 h, 24 h, and 48 h after *F. columnare* infection, PANTHER mapped 4, 61, and 54 genes, respectively. Statistically significant ($P < 0.05$, Bonferroni correction for multiple testing) molecular functions and biological processes of the DAS genes were identified (Fig. 3 and Tables S3-S4). Overall, the overrepresentation tests showed enrichment of RNA binding in molecular functions and RNA splicing in biological processes (Fig. 3).

Table 3
Differential alternative splicing events in channel catfish at each time point after infection with *Flavobacterium columnare* as compared with the control. The two numbers in the parentheses separated by colon () indicate the number of significant alternative splicing events that have higher inclusion levels of the specified events for control sample (first number) or after infection (second number).

	Control vs 4 h	Control vs 24 h	Control vs 48 h
Exon skipping	3 (1:2)	48 (19:29)	47 (17:30)
Mutually exclusive exon	0 (0:0)	4 (0:4)	1 (0:1)
Alternative 5′ splice site	0 (0:0)	12 (4:8)	11 (3:8)
Alternative 3′ splice site	0 (0:0)	0 (0:0)	0 (0:0)
Intron retention	1 (0:1)	10 (9:1)	8 (5:3)
Total	4	74	67

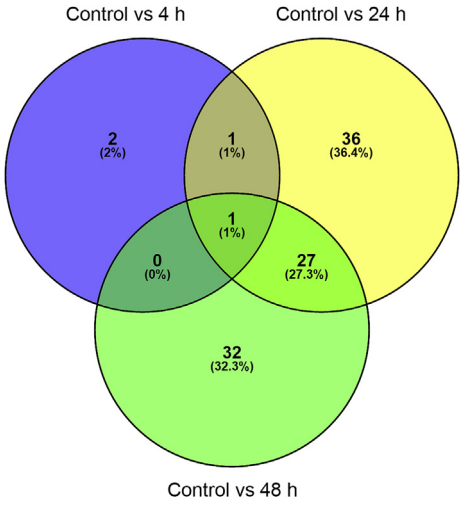

Fig. 2. Venn diagrams showing the distribution of differentially alternatively spliced (DAS) genes among three independent comparisons in channel catfish after *Flavobacterium columnare* infection. Each comparison was performed by analyzing one post-infection sample with the control sample. Percentages of shared and non-shared DAS genes are given and were calculated based on number of total DAS genes after infection.

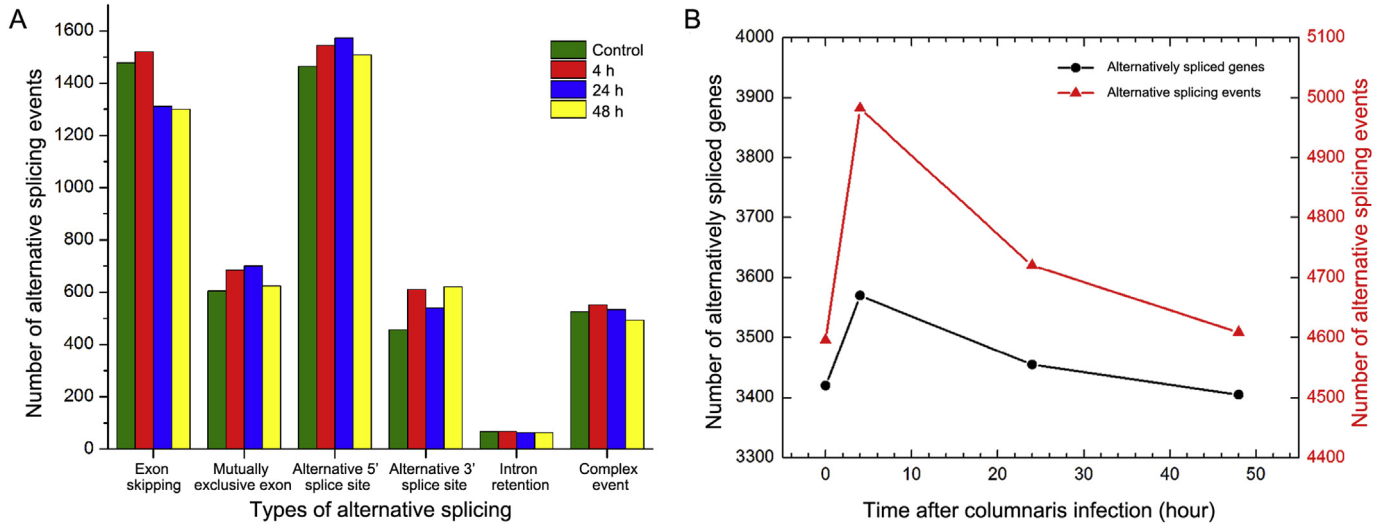

Fig. 1. Changes of alternative splicing in channel catfish after *Flavobacterium columnare* infection, showing the distribution of different alternative splicing types (A), and the number of alternatively spliced genes and alternative splicing events (B) at each time point after *F. columnare* infection. For the control samples, a master pool containing equal amounts of each replicate at all three post-infection time points was formed together and indicated as 0 h.

Table 4
Immune-related differentially alternatively spliced (DAS) genes after infection with *Flavobacterium columnare* in channel catfish. Gene function was collected form UniProt (http://www.uniprot.org), GeneCards (https://www.genecards.org), and Reactome database (http://www.reactome.org).

Gene name	Gene description	Function	Splicing event	Comparison
hectd1	HECT domain E3 ubiquitin protein ligase 1	Class I MHC mediated antigen processing and presentation	Exon skipping	Control vs 24 h; Control vs 48 h
hsh2d	Hematopoietic SH2 domain containing	T cell activation	Alternative 5′ splice site	Control vs 24 h
stom-like	Erythrocyte band 7 integral membrane protein-like	Neutrophil degranulation	Exon skipping	Control vs 24 h
rap1gap2	RAP1 GTPase activating protein 2	Rap1 signaling	Exon skipping	Control vs 24 h
stat3	Signal transducer and activator of transcription 3	Chemokine signaling; JAK-STAT signaling; interleukin-6-mediated signaling	Exon skipping	Control vs 24 h; Control vs 48 h
fbxw2	F-box and WD repeat domain containing 2	Class I MHC mediated antigen processing and presentation	Exon skipping	Control vs 24 h
tcf7	Transcription factor 7	immune response; T-cell lymphocyte differentiation	Exon skipping	Control vs 24 h
hectd1	HECT domain E3 ubiquitin protein ligase 1	Class I MHC mediated antigen processing and presentation	Exon skipping	Control vs 24 h; Control vs 48 h
ubr4	Ubiquitin protein ligase E3 component n-recognin 4	Neutrophil degranulation; Class I MHC mediated antigen processing and presentation	Alternative 5′ splice site	Control vs 48 h
atp6v0a1	ATPase H+ transporting V0 subunit a1	Neutrophil degranulation	Exon skipping	Control vs 48 h
mpo	Myeloperoxidase	Neutrophil degranulation	Intron retention	Control vs 48 h
rnf220	Ring finger protein 220	Class I MHC mediated antigen processing and presentation	Exon skipping	Control vs 48 h

Fig. 3. Enrichment of functional categories in differentially alternatively spliced (DAS) genes in channel catfish after *Flavobacterium columnare* infection. The solid circle and open circle represent the presence or absence, respectively, of certain molecular function or biological process that was enriched in the DAS genes.

4. Discussion

Alternative splicing is a ubiquitous and essential mechanism by which eukaryotes generate remarkable proteomic and regulatory diversity. It provides a critical layer of gene regulation that impacts most, if not all, biological processes. Since the discover of RNA splicing in 1977 [52,53], the identification, characterization and potential role of splicing have interested biologists. Genome-wide profiling of alternative splicing can be achieved by ESTs (expressed sequence tags) and cDNAs [54,55], microarrays [56,57] and RNA-Seq [24,25,58]. RNA-Seq has increasingly become the technology of choice to identify and quantify alternative splicing due to various advantages such as high throughput and the ability to detect novel transcript through deep sequencing [59].

Some progress in understanding the involvement of alternative splicing in the immune system has been made in higher eukaryotes [60]. In contrast, just a few related studies were conducted in fish species. Alternative splicing studies on fish, one of the lower vertebrates, might contribute to the understanding of immune systems evolution as well as the disease control in aquaculture [61,62]. In grass carp (*Ctenopharyngodon idella*), splicing transcripts of immune-related cytokine genes, IL-12p40 and IL-1R1, were differentially expressed in the antiviral response [62]. In our previous study in channel catfish, alternative splicing events and alternatively spliced genes were identified after *Edwardsiella ictaluri* infection [41]. Here we present the alternative splicing analysis after *F. columnare* infection in channel catfish using RNA-Seq datasets, expanding our understanding of the involvement of alternative splicing after bacterial infections.

Alternative splicing is spatially and developmentally regulated and is involved in response to environmental stimuli [63,64]. In this study, the number of alternatively spliced genes and alternative splicing events increased sharply to high levels at the early stage following infection and then decreased to the basal level, reflecting the implication of alternative splicing in acute immune response to *F. columnare* infection. The profile of alternative splicing was similar with that following *E. ictaluri* infection in channel catfish, suggesting a common response pattern of alternative splicing after bacterial infections [41].

Differential alternative splicing can be analyzed at the level of transcripts or splicing events, which focus on differentially expressed transcript isoforms or differential inclusion levels for alternatively spliced transcript regions, respectively. In this study, we used rMATS which utilized likelihood test to identify splicing event-based differential splicing [49]. Compared with the control group, DAS genes were examined at each time point after *F. columnare* in catfish. Shared DAS genes were observed between comparisons (Fig. 2), indicating that the differential splicing of genes was regulated not at only one time point, but at various stages of disease progression. Functional over-representation analysis showed that DAS genes were enriched in RNA

binding and RNA splicing after infection with *F. columnare* (Fig. 3). This was consistent with our previous findings in the study of *E. ictaluri* infection [41]. Furthermore, studies showed that splicing factors themselves were often modulated by alternative splicing in response to environmental conditions [64,65]. Pre-mRNA splicing is mediated by interactions of spliceosome components and a large number of RNA binding proteins with *cis*-sequence elements [66,67]. The abundance and activity of splicing factors or other RNA-interacting proteins play important roles for splicing decisions in physiological responses [68,69]. For example, splicing factor SF3a and SF3b were shown to regulate the alternative splicing of important innate immunity regulators in macrophages of mouse and *Caenorhabditis elegans*, and inhibition of SF3a or SF3b weakened the innate immune response [70,71]. In the present study, splicing factors may be regulated to be alternatively spliced, affecting the downstream immunity regulators in response to *F. columnare* infection. Furthermore, many DAS genes with known immune-related functions were identified (Table 4), despite that immune genes were not enriched using PANTHER. Most of them were implicated in neutrophil degranulation and class I MHC mediated antigen processing/presentation, which play pivotal roles in innate and adaptive immunity, respectively. The involvement of immune-related genes suggested their importance in response to *F. columnare* infection.

In summary, we determined the changes of alternative splicing and indicated functional implication of DAS genes after *F. columnare* infection in channel catfish. The alternative splicing events and involved genes were increased at early stage of response, and enriched functional categories of RNA splicing and RNA binding were significantly enriched in DAS genes after bacterial infection. These findings were consistent with our previous analyses on alternative splicing in response to *E. ictaluri* infection in channel catfish. We suggested that alternative splicing play crucial roles in response to bacterial infections in catfish. Further studies using more tissues and higher sequencing depth will be warranted to provide a more comprehensive analysis, setting a foundation for selecting catfish genotypes with superior performance under adverse environmental conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.008.

Conflicts of interest

The authors declare that they have no conflict of interest.

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