Identification and Characterization of gtf, norB, and trx Genes in Flavobacterium columnare

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Abstract

Three genes from a shotgun genomic library of Flavobacterium columnare virulent strain ALG-00-530 were identified, characterized, and selected for differential expression analysis based on sequence similarity to putative virulence genes from related species. These genes were: glycosyltransferase (gtf), nitric oxide reductase (norB) and thioredoxin (trx). A collection of 30 F. columnare strains, including strains from genomovars I and II, were tested for the presence of these genes. Distribution patterns of gtf, norB, and trx across the species were not uniform. Nucleotide sequence variation was observed between genomovars for each gene; however, strains within the same genomovar shared identical gene sequences. Nine strains of F. columnare, from both genomovars, were chosen for gene expression analysis. The expression profile of these genes varied when selected strains were grown in vitro under identical conditions. ALG-00-530, a high virulent strain, was chosen for gene expression comparison under standard growing conditions, iron-limited conditions and in the presence of skin explants from channel catfish. NorB, and trx gene expression levels varied when ALG-00-530 was incubated under different conditions.

Keywords: Flavobacterium columnare; gtf; norB; trx gene expression; Iron-limited; Catfish skin explants

Introduction

Flavobacterium columnare, the etiologic agent of columnaris disease, is a Gram-negative bacterium that can infect most freshwater fish species. Differences in virulence are known to exist among F. columnare isolates, resulting in variable mortality of fish (Pacha and Ordal, 1970; Suomalainen et al., 2006). Understanding the pathogenesis of F. columnare is critical for fish health, but our knowledge of F. columnare virulence factors is far from being complete.

Only a few genes from F. columnare have been described to date, and most known sequences from this pathogen correspond to ribosomal genes. Among the non-ribosomal sequences identified, the chondroitin AC lyase gene in F. columnare has been previously sequenced and characterized (Xie et al., 2005). The enzyme codified by this gene is able to degrade acidic polysaccharides, such as hyaluronic acid and chondroitin sulfates (Griffin, 1991; Teska, 1993; Stringer-Roth et al., 2002). Differences in chondroitin AC lyase activity have been observed between F. columnare isolates (Stringer-Roth et al., 2002). Relation of chondroitin AC lyase activity to virulence in F. columnare has been recently reported (Suomalainen et al., 2006). Genes encoding outer membrane proteins, such as zinc metalloprotease and prolyl oligopeptidase, have also been characterized in a virulent F. columnare isolate (Xie et al., 2004). In addition, several proteases have been identified in F. columnare, but their specific roles in columnaris pathogenicity are still unknown (Bertolini and Rohovec, 1992; Newton et al., 1997).

The ability of F. columnare to attach to fish tissues is thought to be a factor correlated to virulence (Decostere et al., 1999). Bader et al. (2005) selected for an adhesive-defective F. columnare strain that exhibited reduced virulence to channel catfish. Unfortunately, no adhesin gene has been identified in F. columnare.

Genetic variability of F. columnare species has been characterized using different molecular markers (Triyanto and Wakabayashi, 1999; Arias et al., 2004; Thomas-Jinu and Goodwin, 2004; Darwish and Ismaiel, 2005). Restriction fragment length polymorphism analysis (RFLP) of the 16S rRNA gene divided F. columnare species into three genomovars (Triyanto and Wakabayashi, 1999). The coexistence of three main genomic groups within the species (genomovars I, II, and III) has been corroborated by DNA-DNA hybridization (Triyanto and Wakabayashi, 1999), intergenic spacer region (ISR) sequencing (Darwish and Ismaiel, 2005), and single-strand conformation polymorphism (SSCP) analysis (Olivares-Fuster et al., 2007). To date, most of the genetic markers used for F. columnare strain typing have relied on ribosomal gene analysis.

The aim of this work was to identify and characterize putative virulence genes in this species and to further investigate the genetic diversity of F. columnare using non-ribosomal markers. To achieve this objective, a partially sequenced shotgun genomic library constructed from a virulent F. columnare strain was used. Three putative virulence genes were selected for further analysis. Gene presence and expression patterns were analyzed across the species. Two in vitro experiments aimed to mimic some of the environmental conditions F. columnare encounters during infection (limited iron and fish skin presence) were used for gene expression analysis.

Materials and Methods

Bacterial strains and culture conditions

Thirty strains of F. columnare, representing genomovars I and

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II, were used in this study (Table 1). Cells were cultured in modified Shihe broth (Shoemaker et al., 2005) at 28°C.

**Putative virulence gene identification**

A shotgun genomic library of the *Flavobacterium columnare* virulent strain ALG-00-530 was constructed. Briefly, *Flavobacterium* DNA was extracted and purified following standard protocols (Sambrook and Russell, 2001). Total DNA was partially digested with Sau3A I. Digested DNA ranging from 1 to 1.25 kb was purified by double size selection and ligated to the digested pBlueScript II (KS-) vector (Stratagene, Cedar Creek, TX) at a molar ratio of 1:2. The cloning site was BamHI. Library titer was estimated at 35 recombinant clones per microliter. Quality control tests showed 1% or less empty clones and an expected genomic coverage of 20X. Three thousand clones of this library have been sequenced to date at the USDA-ARS MSA Genomics Laboratory (Stoneville, MS) following standard procedures.

Sequences from the shotgun genomic library were compared with sequences in the GenBank database available at the NCBI (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) using the BLASTX algorithm (Altschul et al., 1990). Sequences with more than 35% identity were recorded. Among the identified genes, three were chosen for further analysis due to their high identity (≥65%) with known genes described as virulence factors in other bacteria. Open reading frames (ORF) of the three sequences were identified using Vector NTI® (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/) using the BLASTP tool (Altschul et al., 1990). Protein-protein identity percentage was recorded for each chosen ORF. Specific primers for each gene were designed using Vector NTI® Suite 8. Primer sequences for each gene were shown in Figure 1. These primers were tested on 30 strains of *Flavobacterium columnare* by PCR. Unless otherwise stated, all PCR reagents were purchased from Promega (Madison, WI). Each 50 µL PCR reaction included 2.5 µM MgCl₂, 1X buffer, 0.2 µM of both primers, 0.2 µM of dNTPs, 1.7 unit of Taq polymerase, and 60 ng of DNA template. The PCR amplification profile was 5 min hot start at 95°C; 35 cycles of 30 s at 94°C, 45 s at 58°C, and 1.5 min at 72°C; and 10 min at 72°C.

**Nucleic acids extraction and RT-PCR**

Total bacterial DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. RNA was extracted from 1.5 mL of bacterial culture using RNeasy plus Mini Kit (Qiagen). Turbo DNA-free kit (Ambion, Austin, TX) was used to eliminate DNA contamination in RNA samples. cDNA synthesis and RT-PCR were performed using the Reverse Transcription System (Promega). One microgram of total RNA was used to synthesize the cDNA in 20 µL of reaction. cDNA synthesis reaction was diluted to 100 µL, and 10 µL were used as template for PCR using gene specific primers, as described above.

| Isolates | Fish species | Geographic origin | G | gtf | norB | trx |
|----------|--------------|-------------------|---|-----|------|-----|
| MS-467   | Channel catfish | Mississippi, USA | I | +   | +    | +   |
| MS-02-465| Channel catfish | Mississippi, USA | I | +   | +    | +   |
| 27       | Channel catfish | Alabama, USA      | I | +   | +    | +   |
| IR       | Common carp   | Israel            | I | +   | +    | +   |
| MS 463   | Channel catfish | Mississippi, USA | I | +   | +    | +   |
| FC-RR    | Channel catfish | Alabama, USA      | I | +   | +    | +   |
| ALG-03-063| Channel catfish | Alabama, USA     | I | +   | +    | +   |
| ALG-03-57 | Channel catfish | Alabama, USA     | I | +   | +    | +   |
| ATCC 23463| Chinook salmon  | Washington, USA   | I | +   | +    | +   |
| ATCC 49512| Brown trout    | France            | I | +   | +    | +   |
| ALG-03-069| Channel catfish | Alabama, USA      | I | +   | +    | +   |
| GA-02-14 | Rainbow trout  | Georgia, USA      | I | -   | +    | +   |
| GZ       | Channel catfish | Alabama, USA      | I | +   | +    | +   |
| ARS-1    | Channel catfish | Alabama, USA      | I | -   | +    | +   |
| BM       | Channel catfish | Alabama, USA      | I | -   | +    | +   |
| HS       | Channel catfish | Alabama, USA      | I | -   | +    | +   |
| MO-02-23 | Largemouth bass | Missouri, USA     | II| +   | +    | +   |
| ALG-00-530| Channel catfish | Alabama, USA     | II| +   | +    | +   |
| LSU      | Channel catfish | Louisiana, USA    | II| +   | +    | +   |
| ALG-00-513| Channel catfish | Alabama, USA      | II| +   | +    | +   |
| PT-14-151| Channel catfish | Mississippi, USA  | II| +   | +    | +   |
| ALG-02-36 | Largemouth bass | Alabama, USA      | II| +   | +    | +   |
| ALG-00-515| Channel catfish | Alabama, USA      | II| +   | +    | +   |
| ALG-00-527| Channel catfish | Alabama, USA      | II| +   | +    | +   |
| MS-475   | Channel catfish | Mississippi, USA  | II| +   | +    | +   |
| ALG-00-522| Channel catfish | Alabama, USA      | II| +   | +    | +   |
| ALG-00-521| Channel catfish | Alabama, USA      | II| +   | +    | +   |
| BZ-1-02  | Nile tilapia   | Brazil            | II| +   | +    | +   |
| BZ-4-02  | Nile tilapia   | Brazil            | II| +   | +    | +   |
| BZ-5-02  | Nile tilapia   | Brazil            | II| +   | +    | +   |

Table 1: Strains of *Flavobacterium columnare* used in the study showing genomovar (G) ascription and standard PCR amplification results of *gtf* (glycosyltransferase gene), *norB* (nitric oxide reductase gene), and *trx* (thioredoxin gene). ‘+’ represents positive PCR amplification of the gene, ‘-’ represents no amplification when DNA was used as template.
Figure 1: Nucleotide sequence alignment of *gtf*, *norB*, and *trx* of *F. columnare* from genomovars I and II (GenBank accession numbers in brackets). 530 stands for ALG-00-530 (genomovar II) and 49512 is the abbreviation of ATCC 49512 (genomovar I). Nucleotides that are different between genomovars are boxed. Sequences used as primers for PCR and RT-PCR amplification are underlined. * indicates putative start codon. Y=C+T, R=A+G, and W=A+T.
Cloning and sequencing analysis

PCR products were resolved through standard agarose gel electrophoresis. Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation, Indianapolis, IN) and cloned into pGEMTEasy (Promega). To ensure sequence accuracy, two clones from each strain were sequenced at the Auburn University Sequencing Core (Auburn, AL). Nucleotide sequence alignments and deduced amino acid sequence alignments were performed by CLUSTAL W algorithm (Chenna et al., 2003).

Gene expression

Expression of gtf, norB, trx was tested under standard conditions, iron-limited conditions, and in the presence of catfish skin explants. Following RNA extraction and cDNA synthesis, genes were amplified from cDNA by the designed primers as mentioned above. 16S rRNA cDNA was amplified as an internal control with universal primers UFL (5'-GGTACACGACGCTCAAGG-3') and UR (5'-CCTAACCGCGGCTGCTT-3') (Chen et al., 2004). Positive (genomic DNA) and negative (total RNA) controls were included for each gene amplification. Amplified products were electrophoresed at 100 V for 30 min and visualized under UV light with ethidium bromide added to a 1% gel to a final concentration of 0.5 µg mL⁻¹.

Gene expression under standard conditions

Nine strains of F. columnare, representing genomovars I and II, were cultured in modified Shieh broth at 28°C for 20 h. Bacterial cells were harvested from one and a half milliliters broth culture followed by RNA extraction and RT-PCR.

ALG-00-530 gene expression under iron-limited conditions

Two iron chelators, 2, 2-dipyridyl and transferrin, were used to remove free iron from the growth medium. 2, 2-dipyridyl was added to 50 mL of Shieh broth to a final concentration of 50 µM. Preparation of the transferrin solution followed the protocol described by Biosca et al., (1993). Briefly, human apo-transferrin (Sigma, St. Louis, MO) was dissolved at a concentration of 1 mg mL⁻¹ in a solution containing 100 µM Tris, 150 µM NaCl, and 50 µM NaHCO₃ (pH 8.0). This solution was sterilized by filtration. Prior to inoculation with F. columnare, transferrin was added to 50 mL Shieh medium to achieve a final concentration of 10 µM and incubated at 37°C for 60 min. Cultured ALG-00-530 bacterial cells were collected by centrifugation at 2,000 g for 20 min. Pellets were washed once in PBS and centrifuged again. Cells were resuspended in approximately 1 mL Shieh broth and then 1/3 mL (of this broth) was transferred into 50 mL Shieh broth containing 2, 2-dipyridyl, or apo-transferrin. Fifty milliliters of Shieh broth was inoculated as control. One and a half milliliters of bacterial cells were collected at time 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h after inoculation. Cells were centrifuged and pellets were resuspended in 100 µL of RNA later (Qiagen) and frozen at -80°C until used. RT-PCR results from nine strains of F. columnare cultured under standard conditions. '–' represents no expression, 'w' represents weak expression, '+' represents expression, '++' represents strongest expression. NA, no amplified product was obtained when genomic DNA was used as template.

| G     | strains    | gtf | norB | trx | 16S rRNA |
|-------|------------|-----|------|-----|----------|
| I     | GA-02-14   | NA  | -    | -   | +++      |
| I     | ARS-1      | NA  | -    | w   | +++      |
| I     | BM         | NA  | -    | +   | +++      |
| I     | HS         | NA  | -    | -   | +++      |
| I     | FC-RR      | -   | +    | -   | +++      |
| II    | BZ-1-02    | -   | -    | +   | +++      |
| II    | LSU        | -   | -    | w   | +++      |
| II    | ALG-00-530 | -   | ++   | ++  | +++      |
| II    | ALG-00-527 | -   | -    | -   | +++      |

Table 2: RT-PCR results from nine strains of F. columnare cultured under standard conditions.
genes as did ALG-00-530 and MS-02-475 genomovar II strains (data not shown). However, nucleotide sequences differed between genomovars (Figure 1). The variation percentage observed was 6.9% for \textit{gtf}, 3.8% for \textit{norB}, and 4.3% for \textit{trx}. Translation of the nucleotide sequences showed the distinction between genomovars was carried out at the protein level. Amino acid sequence variation derived from each gene was not identical between genomovars except for Trx. Gft displayed a 2.2% variation between both genomovars while NorB presented only 0.88% sequence variation.

**Gene expression**

**Gene expression under standard conditions**

The 16S rRNA gene, used as internal control, was expressed at any given time in all experiments. Gene expression patterns of nine strains of \textit{F. columnare} varied at 20 h post inoculation under standard growing conditions and are summarized in Table 2. Gene expression differed not only between genomovars but also within each genomovar. Genomovar I displayed three expression patterns. Strains GA-02-14 and HS shared an identical expression profile, showing no expression of both \textit{norB} and \textit{trx} gene. Strains BM and ARS-1 also shared a similar expression profile with no expression of \textit{norB} but positive expression of \textit{trx}; however, the expression of \textit{trx} gene in BM was stronger than in ARS-1. FC-RR (an avirulent mutant) displayed a distinct pattern with expression of \textit{norB} but no expression of the other two genes. Genomovar II strains showed differences in expression patterns, as well. Stronger expression of \textit{norB} and \textit{trx} was observed in ALG-00-530 compared to other strains. LSU strain showed weak expression of \textit{trx} gene, while BZ-1-02 and ALG-00-527 failed to express any of the tested genes.

**ALG-00-530 gene expression under iron-limited conditions and in the presence of skin explants**

Iron-limited conditions did not interfere with \textit{F. columnare} housekeeping gene expression (Table 3). The 16S rRNA gene was uniformly expressed throughout the experiment. Overall, transferrin seemed to have little effect on \textit{norB} and \textit{trx} gene expression when compared to standard conditions, although \textit{trx} transcript levels seemed lowered when transferrin was incorporated into the medium. 2,2-dipyridyl had an immediate repression effect on \textit{norB} and \textit{trx} expression, while the 16S rRNA gene was not affected by this chemical (Figure 2). However, transcript levels for \textit{norB} and \textit{trx} went back to high levels 2 h after transfer to iron-limited conditions, remaining stable until 8 h and 24 h for \textit{norB} and \textit{trx}, respectively.

When \textit{F. columnare} cells were incubated in cell culture medium with or without skin explants, a distinct expression pattern was observed (Table 3). Little expression of \textit{trx} was detected in both controls and skin explants samples. The \textit{norB} transcript was detected in both samples at different sampling times, although expression levels never reached those observed under standard growing conditions. However, expression levels of the 16S rRNA gene were comparable to those observed under stan-

| Time (h) | gtf | norB | trx | 16S rRNA |
|---------|-----|------|-----|--------|
| In Shieh broth | 0   | -    | ++++| ++++   |
|           | 2   | -    | ++++| ++++   |
|           | 4   | -    | ++++| ++     |
|           | 6   | -    | ++++| ++     |
|           | 8   | -    | ++++| ++     |
|           | 24  | -    | ++++| ++     |
| In the presence of 2,2-dipyridyl | 0   | -    | ++++| ++++   |
|           | 2   | -    | ++++| ++++   |
|           | 4   | -    | ++++| ++     |
|           | 6   | -    | ++++| ++     |
|           | 8   | -    | ++++| ++     |
|           | 24  | -    | ++++| ++     |
| In the presence of transferrin | 0   | -    | ++++| ++     |
|           | 2   | -    | ++++| ++     |
|           | 4   | -    | ++++| ++     |
|           | 6   | -    | ++++| ++     |
|           | 8   | -    | ++++| ++     |
|           | 24  | -    | ++++| ++     |
| C0      | 0   | -    | ++  | +      |
| In the presence of fish skin explants | 0.5 | -    | +   | +      |
|           | 1   | -    | ++  | +      |
|           | 3   | -    | +++| -      |
|           | 6   | -    | ++  | -      |
|           | 10  | -    | +   | +      |
|           | 24  | -    | +   | +      |
| Controls for the fish skin explants | 0.5 | -    | ++  | +      |
|           | 1   | -    | ++  | -      |
|           | 3   | -    | +++| -      |
|           | 6   | -    | +   | +      |
|           | 10  | -    | +   | +      |

**Table 3:** \textit{F. columnare} ALG-00-530 gene expression under standard conditions, iron-limited conditions, and in the presence of catfish skin explants. '-' represents no expression, relative intensity of the expressed genes is expressed by the number of '+' symbols.
Prior to the experiment, bacterial broth at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. 16S rDNA (16S) was used as an internal control. Expression patterns are shown under iron-limited conditions in the presence of 2, 2-dipyridyl and in Shieh broth at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. 16S rDNA (16S) was used as an internal control.

Figure 2: HemH, norB, and trx gene expression of F. columnare strain ALG-00-530 by reverse transcription PCR (RT-PCR). Expression patterns are shown under iron-limited conditions in the presence of 2, 2-dipyridyl and in Shieh broth at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. 16S rDNA (16S) was used as an internal control.

Discussion

F. columnare has been reported to be a morphological (Song et al., 1998; Thomas-Jinu and Goodwin, 2004), serological (Anacker and Ordal, 1959), and genomically (Toyama et al., 1999; Triyanto and Wakabayashi, 1999; Arias et al., 2004; Thomas-Jinu and Goodwin, 2004; Darwish and Ismaiel, 2005; Schneck and Caslake, 2006) diverse species. According to Bernardet and Grimont, (1989), as some F. columnare strains shared only a 78% similarity by DNA-DNA hybridization. Triyanto and Wakabayashi, (1999) described three different genomovars among the species based on the analysis of the 16S rRNA-RFLP. Genomovar ascription has become routine for F. columnare strain characterization (Triyanto and Wakabayashi, 1999; Arias et al., 2004; Olivares-Fuster et al., 2007). However, beyond ribosomal variability, no study has been conducted to investigate the intra-species variation of F. columnare at the single gene level, mainly due to the lack of genetic information available for this species.

In the current study, glycosyltransferase gene (gtf), nitric oxide reductase gene (norB), and thioredoxin gene (trx) have, for the first time, been identified and characterized in F. columnare. These genes presented a high similarity to homologous genes within the Flavobacterium genus, showing the highest similarity with F. johnsoniae gene sequences. Although strains within the same genomovar showed identical gene sequences, there was a 4 to 7% nucleotide sequence variation observed between genomovars for each gene. The variability found at the nucleotide level was also translated to the amino acid level. Lee et al., (1998) suggested that single amino acid substitutions could change the biological activity of proteins. We found some non-conserved amino acid substitutions between the genomovar I ALG-00-530 and the genomovar II TCC 49512 in Gtf, and NorB protein sequences (data not shown). Therefore, the activity of these proteins could differ between F. columnare genomovars.

Our data agreed with the genomovar segregation previously reported for F. columnare. Some examples have been published of such correlation between ribosomal gene-based variability and non-ribosomal gene-based variability i.e. F. psychrophilum (Soule et al., 2005). Non-ribosomal variation divided F. psychrophilum into two lineages that are associated with different host species. This is not the case for F. columnare, since channel catfish is susceptible to both F. columnare genomovars. However, preliminary studies showed genomovar II strains were more virulent to channel catfish than genomovar I strains (Craig A. Shoemaker, USDA-ARS, Auburn, AL, personal communication).

Gtf, norB, and trx genes characterized in the present study have been described as virulence factors in other bacterial species (Almiron et al., 2001; Narimatsu et al., 2004; Bjr et al., 2006; Loisel-Meyer et al., 2006). Our expression data showed that norB and trx genes were weakly or not expressed when cells reached the end of the log phase approximately 24 h growth; however, they were strongly expressed during the first few hours following inoculation into fresh Shieh broth culture medium. Both norB and trx gene expression in F. columnare was not inhibited under iron-limited conditions. Holmes et al., (2005) reported that iron-limited conditions actually increased the transcript level of thioredoxin in Campylobacter jejuni.Iron-limited conditions also could lead to oxidative stress in Anabaena sp. (Latifi et al., 2005). Additionally, both norB and trx can defend against oxidative and/or NO-mediated stress (Wieles et al., 1997; Jaegera et al., 2004; Philippot, 2005; McGee et al., 2006). As a result, it is possible that F. columnare continues to express both norB and trx in order to reduce oxidative stress due to iron deficiency or limitation.

Gene expression was weak when cells were transferred to cell culture medium, regardless of the presence of catfish skin explants. This may be the result of the relative high salinity in this medium. During the catfish skin explants experiment, bacterial cells were cultivated in cell culture medium 199 diluted 1:1 with bacterial broth (final salinity was about 0.45%). This salinity was required to keep the skin cells alive. It has been reported that increased salinity significantly reduced growth and adhesion ability of F. columnare (Altinok and Grizzle, 2001). The general trend observed was a reduction in gene expression that might be due to the high salinity levels; however, expression levels of the 16S rRNA gene seemed to be unaffected.

In conclusion, our data confirmed the genomic diversity of F. columnare at the single gene level. Nucleotide sequences of gtf, norB, and trx of F. columnare differed between genomovars I and II. Multiple gene expression patterns existed both between and within genomovars. Although the relation between gene expression pattern and virulence is unclear, this study addressed for the first time in vitro gene expression in F. columnare. Further studies are ongoing in order to confirm the difference in virulence between genomovars I and II. Nevertheless, and due to the clear genetic division between genomovars, we strongly recommend the inclusion of more than one genomovar in future F. columnare studies.

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