Journal of Microbial & Biochemical Technology - Open Access
JMBT/Vol.1 Issue 1

Research Article | OPEN ACCESS Freely available online | doi:10.4172/1948-5948.1000013

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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Received December 10, 2009; **Accepted** December 26, 2009; **Published** December 26, 2009

Citation: Zhang Y, Arias CR (2009) Identification and Characterization of gtf, norB, and trx Genes in Flavobacterium *columnare*. J Microb Biochem Technol 1: 064-071. doi:10.4172/1948-5948.1000013

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

Putative virulence gene identification

A shotgun genomic library of the *F. columnare* virulent strain ALG-00-530 was constructed. Briefly, *F. columnare* DNA was extracted and purified following standard protocols (Sambrook and Russell, 2001). Total DNA was partially digested with *Sau*3A 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 *Bam*H I. 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® Suite 8 software package (Invitrogen, Carlsbad, CA). ORF were translated into amino acid sequences by using Vector NTI® Suite 8 and the GenBank database was searched for protein sequences 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 *F. columnare* by PCR. Unless otherwise stated, all PCR reagents were purchased from Promega (Madison, WI). Each 50 µL PCR reaction included 2.5 µM $MgCl_2$, 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 95ºC; 35 cycles of 30 s at 94ºC, 45 s at 58ºC, and 1.5 min at 72ºC; and 10 min at 72ºC.

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 *F. 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.

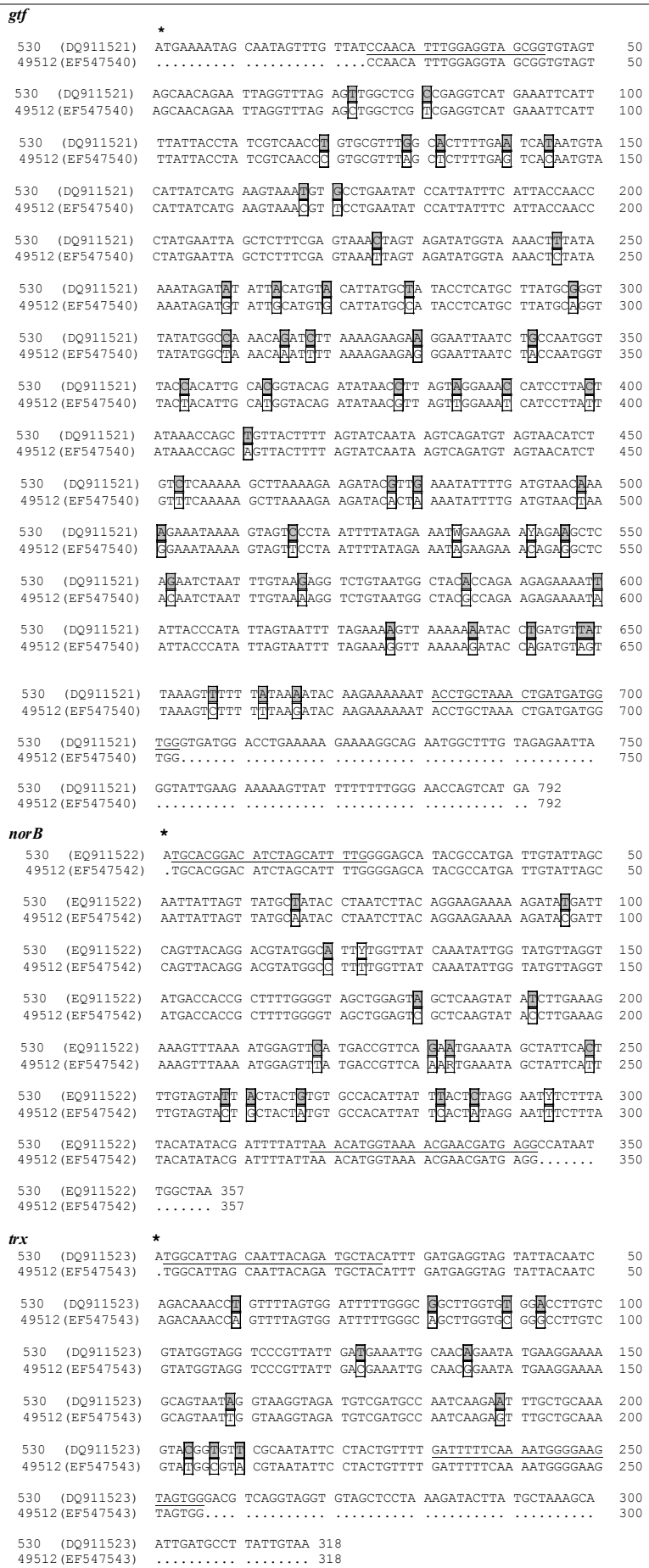

Figure 1: Nucleotide sequence alignment of *gtf*, *norB*, and *trx* of *F. columnare* from genomovars I and II (GenBank assession numbers in brackets). 530 stands for ALG-00-530 (genomovar II) and 49512 is the abbreviation of ATCC 49512 (genomovar I). Nucleotides that are different between genomovars are boxed. Sequences used as primers for PCR and RT-PCR amplification are underlined. * indicates putative start condon. 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 UFUL (5'-GCCTAACACAT GCAAGTCGA-3') and URUL (5'-CGTATTACCGCGGCT GCTGG-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 28ºC 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 μM in a solution containing 100 μM Tris, 150 μM NaCl, and 50 μM $NaHCO_3$ (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 37ºC 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 -80ºC until used. RT-PCR was performed as explained above.

ALG-00-530 gene expression in the presence of catfish skin explants

Skin explants from channel catfish that tested negative for *F. columnare* antibody presence were obtained according to the procedure by Xu and Klesius, (2002). One milliliter of Medium 199 (Sigma) supplemented with fetal bovine serum at 2.5% was added into each well of a 24-well culture plate (Costar, Cambridge, MA). One cm^2 of fish skin explant was placed onto the bottom of 12 wells. One and a half milliliters of an overnight culture of ALG-00-530 bacterial cells was collected for the baseline control (C_0); one and half milliliters of the same bacterial culture was added into each of the 24 wells. Wells without fish skin explants were regarded as controls, while wells containing the fish explants were referred to as treatments. Bacterial cells were collected from each individual well at 30 min, 1 h, 3 h, 6 h, 10 h, and 24 h from both control and treatment wells. After centrifugation, bacterial pellets were suspended in RNA later (Ambion, Austin, TX) and frozen at -80ºC until used. RNA extraction and RT-PCR were performed as described above.

Results

Putative virulence gene identification

Out of 3,000 clones sequenced from the ALG-00-530 shotgun library, 73 sequences displayed a high nucleotide similarity with known genes present in GenBank (based on BLASTX search, data not shown). Within this subset, three sequences were identified from published literature as putative virulence genes and therefore selected for this study: glycosyltransferase gene (*gtf*) (GenBank accession number DQ911521), nitric oxide reductase gene (*norB*) (GenBank accession number DQ911522), and thioredoxin gene (*trx*) (GenBank accession number DQ911523). ORF were translated into amino acid sequences which were then compared to GenBank sequences using BLASTP. Identity percentages of ORF based on protein-protein alignment were as follows: Gtf was 81% identical to *F. johnsoniae* sequence accession number ZP_01245614, NorB was 77% identical to an unidentified *Flavobacterium* species sequence accession number ZP_01106280, while Trx was 83% identical to *F. johnsoniae* sequence accession number ZP_01243853. Based on the PCR results, all strains contained *norB* and *trx* genes (Table 1). However, no PCR amplification for *gtf* was observed in four strains (GA-02-14, ARS-1, BM, and HS).

Cloning and sequencing analysis

Two *F. columnare* strains from genomovar I (ATCC 49512 and ALG-03-57) and genomovar II (ALG-00-530 and MS-02-475) were selected for multi locus sequence analysis using *gtf*, *norB*, and *trx* genes. Nearly complete sequences were amplified from *gtf*, *norB*, and *trx* and used for comparison. Alignment of these sequences showed two distinct populations. Both genomovar I strains shared an identical sequence for all three

G	strains	*gtf*	*norB*	*trx*	*16SrRNA*
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. G represents genomovar ascription. '–'represents no expression, 'w' represents weak expression, '+' represents expression, '++' represents strong expression. '+++' represents strongest expression, NA, no amplified product was obtained when genomic DNA was used as template.

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 *gtf*, 3.8% for *norB*, and 4.3% for *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 *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 *norB* and *trx* gene. Strains BM and ARS-1 also shared a similar expression profile with no expression of *norB* but positive expression of *trx*; however, the expression of *trx* gene in BM was stronger than in ARS-1. FC-RR (an avirulent mutant) displayed a distinct pattern with expression of *norB* but no expression of the other two genes. Genomovar II strains showed differences in expression patterns, as well. Stronger expression of *norB* and *trx* was observed in ALG-00-530 compared to other strains. LSU strain showed weak expression of *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 *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 *norB* and *trx* gene expression when compared to standard conditions, although *trx* transcript levels seemed lowered when transferrin was incorporated into the medium. 2, 2-dipyridyl had an immediate repression effect on *norB* and *trx* expression, while the 16S rRNA gene was not affected by this chemical (Figure 2). However, transcript levels for *norB* and *trx* went back to high levels 2 h after transfer to iron-limited conditions, remaining stable until 8 h and 24 h for *norB* and *trx*, respectively.

When *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 *trx* was detected in both controls and skin explants samples. The *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-dipirydyl	0	-	+	+	+++
	2	-	++++	+++	+++
	4	-	+++	++	++
	6	-	+++	+	++
	8	-	+++	+++	+++
	24	-	-	++++	+++
In the presence of transferrin	0	-	++++	+	+++
	2	-	++++	++	+++
	4	-	+++	++	+++
	6	-	-	-	++
	8	-	+	+	+++
	24	-	+	+	+++
C_0	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: *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.

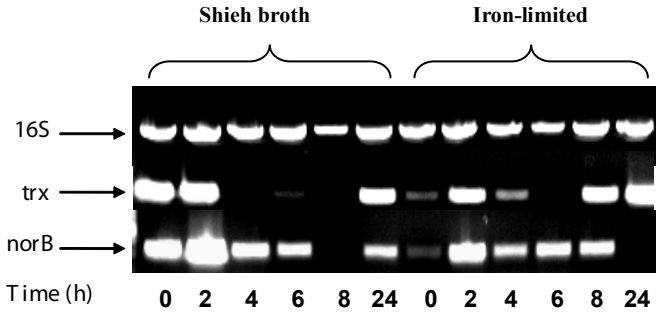

Figure 2: *HemH*, *norB*, and *trx* gene expression of *F. columnare* strain ALG-00-530 by reverse transcription PCR (RT-PCR). Expression patterns are shown under iron-limited conditions in the presence of 2, 2-dipyridyl and in Shieh broth at 0 h, 2 h, 4 h, 6 h, 8 h, and 24 h. 16S rDNA (16S) was used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

dard conditions. Prior exposure to the culture medium, *F. columnare* cells cultivated overnight displayed the same pattern of expression previously observed under normal growing conditions.

Discussion

F. columnare has been reported to be a morphological (Song et al., 1988; Thomas-Jinu and Goodwin, 2004), serological (Anacker and Ordal, 1959), and genomically (Toyama et al., 1996; 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 rDNA-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 ATCC 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; Bjur et al., 2006; Loisel-Meyer et al., 2006). Our expression data showed that *norB*, and *trx* genes are 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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