

## Identification of a multidrug efflux pump in *Flavobacterium johnsoniae*

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**Abstract** – In this study, the mechanism conferring multiple drug resistance in several strains of flavobacteria isolated from the ovarian fluids of hatchery reared 3-year old brook trout *Salvelinus fontinalis* was investigated. Metabolic fingerprinting and 16S rRNA gene sequences identified the isolates as *Flavobacterium johnsoniae*. The isolates exhibited multiple resistances to a wide range of antimicrobial classes including penicillin, cephem, monobactam, aminoglycoside, and phenicol. Although plasmids and other transposable elements containing antimicrobial resistance genes were not detected, the isolates did contain a genomic sequence for a chloramphenicol-inducible resistance-nodulation-division family multidrug efflux pump system. Efflux pumps are non-specific multidrug efflux systems. They are also a component of cell-cell communication systems, and respond specifically to cell membrane stressors such as oxidative or nitrosative stress. Understanding of efflux pump mediated antibiotic resistances will affect efficacy of clinical treatments of fishes associated with *F. johnsoniae* epizootics.

***Flavobacterium johnsoniae* / multidrug efflux pump / antibiotic resistance / aquaculture / brook trout**

### 1. INTRODUCTION

*Flavobacterium* spp. are Gram-negative, oxidase-positive, indole-negative, yellow-pigmented, and non-fermenting rods with gliding motility which are common worldwide in aquatic environments [6, 23, 38]. Several species, including *F. johnsoniae*, *F. psychrophilum*, *F. branchiophilum*, and *F. columnare*, have been associated with clinical disease in fishes and represent major threats to commercial aquaculture [7, 13–15]. Prevention of flavobacteria epizootics remains difficult due to the ubiquitous presence of *Flavobacterium* spp. in

soils and waters [45, 46]. Therefore, prudent aquaculturists employ immaculate hygiene, low fish densities, and proper feeding rates as the primary defenses against opportunistic *Flavobacterium* outbreaks. Short-term, government approved antimicrobial drugs provide effective chemotherapy against some *Flavobacterium* isolates; however, the identification of multidrug resistant (MDR) strains indicates that the control of disease outbreaks caused by *Flavobacterium* spp. will remain a significant challenge [2, 8, 9, 40].

The appropriate choice of effective antimicrobial agents for treatment of *Flavobacterium* spp. is difficult due to their decreased susceptibility to many antimicrobial agents routinely

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used in aquacultural environments [40]. The mechanisms for these drug resistances in flavobacteria are not well characterized. We reported that MDR and mercury resistant *Aeromonas salmonicida* in clinically diseased Atlantic salmon *Salmo salar* was associated with a conjugative Inc A/C plasmid closely resembling the PSN254 plasmid from the human pathogen *Salmonella enterica* [27]. Plasmids have been reported in *Flavobacterium* [11, 43] and antimicrobial resistance genes have been identified in *Flavobacterium* and *Chryseobacterium* [3, 11, 18, 37, 43], but the presence of these genes only partially accounts for the MDR phenotypes observed in these isolates from diseased salmonids. Intrinsic antibiotic resistance in several species of Gram-negative bacteria has been attributed to low permeability of the outer membrane, and to efflux pump mechanisms [21, 31, 39]. The contribution of the resistance-nodulation-division (RND) family of antimicrobial efflux pumps to intrinsic antibiotic resistance has been identified in a number of Gram-negative bacteria, including *Bacteroides*, *Chryseobacterium*, *Pseudomonas*, *Salmonella*, *Campylobacter*, and *Vibrio* [17, 29, 32–35, 44]. RND multidrug efflux pumps are composed of a tripartite system that includes a cytoplasmic membrane RND pump, a periplasmic membrane fusion protein, and an outer membrane protein [34]. Unlike other systems that confer resistance to specific antimicrobial compounds or to classes of antibiotics, RND efflux pumps are non-specific multidrug efflux systems. RND efflux pumps are also a component of cell-cell communication systems, and they respond specifically to cell membrane stressors such as oxidative or nitrosative compounds [34].

In this study, we identify a chloramphenicol-inducible RND multidrug efflux pump system in *F. johnsoniae*. RND efflux pump genes identified in this study are encoded on the *Flavobacterium* spp. chromosome, and not on mobile genetic elements, such as transposons or plasmids. This is the first report of an RND multidrug efflux pump in the genus *Flavobacterium* and is significant because RND pumps can influence pathogenesis and can function as virulence determinants in this fish pathogen [34].

## 2. MATERIALS AND METHODS

### 2.1. Flavobacteria isolate identification

Seven flavobacteria isolates were obtained from the ovarian fluids of 3-year old spawning female brook trout in Maine, USA, fish hatcheries. Samples were collected from females with abnormally cloudy, coagulating ovarian fluid and less than 20% egg survival. The isolate designations were P545, P547, P550, G873, G888, G892, and G899. Flavobacteria isolates were selected from colonies initially grown on tryptic soy agar (TSA) plating media (Difco-BBL, Sparks, MD, USA) at room temperature (22 °C). All pure cultures were Gram-negative, weakly motile in wet mount preparations, oxidase-positive, indole-negative, yellow-pigmented, and non-fermenting rods.

Flavobacteria isolates were further identified and characterized from genus to species by the Aquatic Animal Health Laboratory (University of Maine-Orono, USA) using the Biolog GN2 MicroPlate Identification System (Biolog, Hayward, CA, USA). Each isolate was independently identified by comparing 16S rRNA gene sequence data using *F. johnsoniae* 17061<sup>T</sup> (ATCC 17061) as a positive control. Genomic DNA extraction of all isolates was performed using the E.Z.N.A.<sup>®</sup> Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. A fragment of the 16S rRNA gene sequence, approximately 1.3 kb in size, was amplified in each strain using the primers 27F and 1392R [20]. Amplified products were purified using the E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek), and sequenced using the Big Dye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA). GenBank database searches (August, 2008) were carried out for all sequences using the National Center for Biotechnology Information basic local alignment search tool (BLAST) web server<sup>1</sup>. Multiple sequence alignments were performed using the ClustalW alignment function of the MacVector 10.0 software package (MacVector, Inc., Cary, NC, USA) to verify the strain specificity of each *Flavobacterium* isolate.

### 2.2. Antibiotic susceptibility testing

Antibiotic Minimum Inhibitory Concentrations (MIC) were determined using the Sensititre<sup>®</sup> GN2F and GPN2F Panel (Trek Diagnostic Systems, Westlake, OH, USA) dried susceptibility panels.

<sup>1</sup> <http://www.ncbi.nlm.nih.gov/BLAST>.

Manufacturer's instructions were followed with the exception that isolates were incubated at 22 °C for 48 h. Antibiotic MIC assays were done in triplicate (Tab. I). MIC values were determined as the lowest concentration of the antimicrobial agent able to inhibit growth. As recommended by Clinical and Laboratory Standards Institute and Sensititre® guidelines [12], the following reference strains were included as internal standards: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterococcus faecalis* (ATCC 29212). Initial testing determined that the MIC value for the efflux pump inhibitor Phenylalanine-Arginine- $\beta$ -naphthylamide (Pa $\beta$ N) (Sigma-Aldrich, St. Louis, MO, USA) was 200  $\mu$ g/mL for all strains. A final Pa $\beta$ N concentration of 20  $\mu$ g/mL was used as indicated (Tab. I).

### 2.3. Plasmid profiling

Plasmid extractions were performed on all isolates using the Qiagen Plasmid Mini Kit and the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions and as previously reported [27]. An *E. coli* DH5- $\alpha$ <sup>TM</sup> strain (Invitrogen, Carlsbad, CA, USA), containing the pUC19 vector (New England BioLabs®, Ipswich, MA, USA), was used as the positive plasmid control and the plasmid-less strain, *E. coli* DH5- $\alpha$ <sup>TM</sup>, was used as the negative control.

### 2.4. Cloning of antibiotic-resistance genes and analysis of recombinant plasmids

Genomic DNA was extracted from *F. johnsoniae* G873 as described above and partially digested with *Sau*3AI (Promega, Madison, WI, USA). Genomic DNA fragments were ligated into the phagemid pBK-CMV (Stratagene®, La Jolla, CA, USA), previously digested with *Bam*HI (New England BioLabs®), and dephosphorylated with calf intestinal alkaline phosphatase (New England BioLabs®). Ligations were performed using the Fast-Link<sup>TM</sup> DNA Ligation Kit (Epicentre® Biotechnologies, Madison, WI, USA), according to manufacturer's instructions. Recombinant plasmids were transformed into *E. coli* NEB 5- $\alpha$  competent cells (New England BioLabs®). Antibiotic-resistant colonies were selected on plates of TSA containing: ampicillin (16  $\mu$ g/mL), amikacin (30  $\mu$ g/mL), gentamycin (30  $\mu$ g/mL), kanamycin (30  $\mu$ g/mL), streptomycin (30  $\mu$ g/mL), or chloramphenicol (16  $\mu$ g/mL). Antibiotics were purchased from Sigma-Aldrich. Following plasmid isolation from resistant clones, vector inserts

were sequenced using the pBK-CMV vector primers T3 and T7 (Stratagene 2008), designed by Stratagene. Sequences were entered into GenBank database searches (August, 2008) using the BLAST web server for identification.

### 2.5. Identification of MexB and TonB homologs

A BLAST protein homology search was conducted using the *F. johnsoniae* 17061<sup>T</sup> complete genomic sequence, submitted to GenBank by the DOE Joint Genome Institute (CP000685), with the amino acid sequence of MexB (*P. aeruginosa* PA01 MexB, accession number NP\_249117) as a query. Matches with a 37% or greater identity were considered for further analysis.

PCR primers to screen the *F. johnsoniae* isolates for the identified *mexB* homologs were designed from the *F. johnsoniae* UW101 gene sequences using the Primer 3 software program<sup>2</sup>. Additional primers were designed in the same manner to screen for a gene encoding a TonB family protein (Fjoh\_0490, accession number YP\_01192844) present in *F. johnsoniae* UW101. Primers for the corresponding *mexA* (Fjoh\_4299, accession number YP\_001196626) and *oprM* (Fjoh\_4301, accession number YP\_001196628) homologs of the *mexB* homolog Fjoh\_4300 (accession number YP\_001196627) in *F. johnsoniae* UW101 were also designed to screen the *F. johnsoniae* isolates. All PCR primers used are listed in Table II. Following the sequencing of amplification products, sequences were entered in GenBank database searches (August, 2008) to determine identity matches, and multiple sequence alignments were performed using the ClustalW alignment function of the MacVector 10.0 software package.

### 2.6. Quantitative real-time reverse transcription-PCR (qRT-PCR)

Liquid cultures of *F. johnsoniae* G873 in 50 mL LB containing sub-inhibitory concentrations of chloramphenicol (4 to 8  $\mu$ g/mL), norfloxacin (1 to 3  $\mu$ g/mL), ampicillin (10  $\mu$ g/mL) or ethidium bromide (2 to 50  $\mu$ g/mL) were statically incubated at room temperature overnight. Total RNA was extracted using the RNeasy® Protect Bacteria Mini Kit (Qiagen). Gene expression was quantified by a one-step real-time RT-PCR performed on an Applied Biosystems

<sup>2</sup> [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

**Table I.** Susceptibility of *F. johnsoniae* G873 to antimicrobial agents in the absence and presence of the efflux pump inhibitor PaβN.

Class	Antimicrobial agent	MIC plate dilution range ( $\mu\text{g/mL}$ )	MIC ( $\mu\text{g/mL}$ )	
			0 $\mu\text{g/mL}$ PaβN	20 $\mu\text{g/mL}$ PaβN
<b>Penicillin</b>				
	Ampicillin	GPN2F (0.12–16)	16	8
	Ampicillin/sulbactam	GN2F (4/2–32/16)	16/8	8/4
	Oxacillin + 2% NaCl	GPN2F (0.25–8)	0.25	0.25
	Penicillin	GPN2F (0.06–8)	8	4
<b>Cephem</b>				
	Cefazolin	GN2F (4–32)	> 32	> 32
	Cefepime	GN2F (4–32)	8	8
	Ceftazidime	GN2F (1–32)	> 32	> 32
<b>Macrolide</b>				
	Erythromycin	GPN2F (0.25–8)	4	0.5*
	Clarithromycin	GPN2F (1–8)	1	1
<b>Monobactam</b>				
	Aztreonam	GN2F (8–32)	> 32	> 32
<b>Lincosamide</b>				
	Clindamycin	GPN2F (0.5–4)	0.5	0.5
<b>Fluoroquinolone</b>				
	Levofloxacin	GPN2F (0.25–8)	1	0.25*
<b>Oxazolidinone</b>				
	Linezolid	GPN2F (0.5–8)	4	1*
<b>Streptogramin</b>				
	Quinupristin/dalfopristin	GPN2F (0.25–4)	> 4	4
<b>Ansamycin</b>				
	Rifampin	GPN2F (0.5–4)	0.5	0.5
<b>Tetracycline</b>				
	Tetracycline	GPN2F (2–16)	2	2
<b>Aminoglycoside</b>				
	Amikacin	Dilution assay	140	100
	Gentamicin	Dilution assay	140	100
	Kanamycin	Dilution assay	450	400
	Streptomycin	Dilution assay	64	32
<b>Phenicol</b>				
	Chloramphenicol	Dilution assay	16	4*
	Florfenicol	Dilution assay	16	4*
<b>Fluoroquinolone</b>				
	Nalidixic acid	Dilution assay	140	140
	Norfloxacin	Dilution assay	4	1*

\* Four-fold MIC decrease; GPN2F Trek Diagnostic Systems Sensititre Gram-Positive MIC Plate; GN2F Trek Diagnostic Systems Sensititre Gram-Negative MIC Plate.

StepOne™ Real-Time PCR System (Foster City, CA, USA) using the QuantiFast SYBR® Green RT-PCR Kit (Qiagen). PCR primers were designed as described

above (Tab. II). RNA expression in samples (100 ng/ $\mu\text{g}$ ) was normalized using 16S rRNA (1 ng/ $\mu\text{L}$ ), as done previously for *Flavobacterium* [16].

**Table II.** Primers used in this study were designed from *F. johnsoniae* 17061<sup>T</sup>. All Fjoh primers are based on the *mexB* homolog sequences with the exception of Fjoh\_4299 and Fjoh\_4301, which are based on the *mexA* and *oprM* homolog sequences.

Name	Sequence	Target	Reference
27F	AGAGTTTGATCCTGGCTCAG	16S rRNA	[20]
1392R	GACGGGCGGTGTGTAC	16S rRNA	[20]
16SR	TAGCACGTGTGTAGCCCAAG	16S rRNA, for qRT-PCR	This study
16SF	CGCAACCCCTGTTGTTAGTT	16S rRNA, for qRT-PCR	This study
T3	AATTAACCCCTACTAAAGG	PBK-CMV plasmid insert	[41]
T7	GTAATACGACTCACTATAGGGC	PBK-CMV plasmid insert	[41]
0907R	TCAGGCACCGTCAAATTGTA	Fjoh_0907	This study
0907F	TTTGGCGGTATAAAAAGGAG	Fjoh_0907	This study
4131R	AAGAAGCCCGATAAGCATGA	Fjoh_4131	This study
4131F	TCGATTCCGTTTGGATTAGC	Fjoh_4131	This study
4300R	CCATTTCGCCTGTTTTGTTT	Fjoh_4300	This study
4300F1	CGCACAGGCATCTGACTTTA	Fjoh_4300	This study
4300F2	CCAAGTGTCCCGGATTTAG	Fjoh_4300	This study
4299R	TGTC AAGTTTCCGCTTACC	Fjoh_4299	This study
4299F	TCCCGAGAATGCCAGAATAC	Fjoh_4299	This study
4301R	TTGCTTGTGCTTGC GTTTAC	Fjoh_4301	This study
4301F	AACCAAAATCGTGACCTTCG	Fjoh_4301	This study
3239R	CAGAATCGTTCCGTCTGGAT	Fjoh_3239	This study
3239F	GATCCGGACAGCTTGACAAT	Fjoh_3239	This study
4862R	GCACTTTGTGCGATGATGTT	Fjoh_4862	This study
4862F	CGATTTTGGCCAATACATTT	Fjoh_4862	This study
CFLR	ACAGCTTCCGATAGCCTGAA	Cfl/Ber efflux pump	This study
CFLF	AACCGCTCTTGGTCTTTTT	Cfl/Ber efflux pump	This study

## 2.7. Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been assigned to GenBank databases and assigned accession numbers EU984145-EU984170 and EU984179-EU984187.

## 3. RESULTS

### 3.1. *Flavobacterium* spp. identification

All *Flavobacterium* isolates, except P547, were identified as 100% probability matches to *F. johnsoniae* through metabolic fingerprinting using the Biolog GN2 MicroPlate Identification System. Isolate P547 was identified as *F. johnsoniae*, however the probability could not be determined because this isolate's metabolic fingerprint also closely matched the fingerprint of *F. hydatis*.

The 1.3 kb 16S rRNA gene sequences for all isolates and the *F. johnsoniae* 17061<sup>T</sup> positive control matched the *F. hydatis* GenBank database strain DSM 2063T with 98% sequence similarity. *F. johnsoniae* and *F. hydatis* are very closely related phylogenetically and almost impossible to discriminate, even by genomic approaches. Based on the Biolog GN2 results and 16SrRNA gene sequence data, the flavobacteria isolates were presumptively identified as *F. johnsoniae*.

### 3.2. Antimicrobial susceptibility

Antibiotic susceptibility testing indicated multidrug resistance profiles for all *F. johnsoniae* isolates were similar. *F. johnsoniae* G873 isolate for example, showed susceptibility to oxacillin + 2% NaCl, clarithromycin, clindamycin, rifampin, and tetracycline, and resistance to cefazolin, ceftazidime, aztreonam, quinupristin/

dalfoprisin, chloramphenicol and florfenicol (Tab. I). Susceptibility data of other isolates are not shown because they were similar. Isolate G873 was selected for subsequent antimicrobial susceptibility testing with Pa $\beta$ N. The remaining isolates were archived.

### 3.3. Identification of genomic antimicrobial resistance genes

Plasmids were not detectable in any of the *F. johnsoniae* isolates. An initial attempt to determine the presence of antibiotic-resistance genes in the *F. johnsoniae* isolates utilized genomic libraries, which, following construction, were used to screen for antibiotic-resistance determinants. Antibiotic selection of the genomic library of strain *F. johnsoniae* G873 resulted in the growth of an amikacin-resistant clone. When sequenced, this clone encoded a sequence with an 85% identity match to the nucleotide sequence of a gene encoding a TonB-dependent receptor in *F. johnsoniae* UW101 (Fjoh\_1451, accession number YP\_001193802). In order to verify the presence of *tonB* in *F. johnsoniae* G873 as well as in the other *F. johnsoniae* isolates, a partial nucleotide sequence for a gene that encodes a TonB family protein in *F. johnsoniae* UW101 (Fjoh\_0490, accession number YP\_01192844) was amplified in all isolates.

### 3.4. MexB RND efflux pump homologs in *F. johnsoniae* isolates

Genomic analysis of *F. johnsoniae* UW101 resulted in the identification of seven *P. aeruginosa* PA01 MexB RND efflux pump homolog proteins with 37–41% sequence similarity. These MexB homologs include Fjoh\_4300 (accession number YP\_001196627), Fjoh\_4131 (accession number YP\_00119648), Fjoh\_3239 (accession number YP\_001195574), Fjoh\_4862 (accession number YP\_001197180), Fjoh\_0907 (accession number YP\_001193260), Fjoh\_4408 (accession number YP\_001196733), and Fjoh\_3227 (accession number YP\_001195563).

Interestingly, each of the *mexB* homologs was associated with *mexA* and *oprM* homologs in the same gene arrangement as the

*mexAB-oprM* operon. Partial genetic regions of five of these *mexB* homologs, including Fjoh\_4300, Fjoh\_4131, Fjoh\_3239, Fjoh\_4862, and Fjoh\_0907, were successfully amplified in *F. johnsoniae* G873. The primer sets for these sequences were subsequently used for the qRT-PCR experiments (Tab. II).

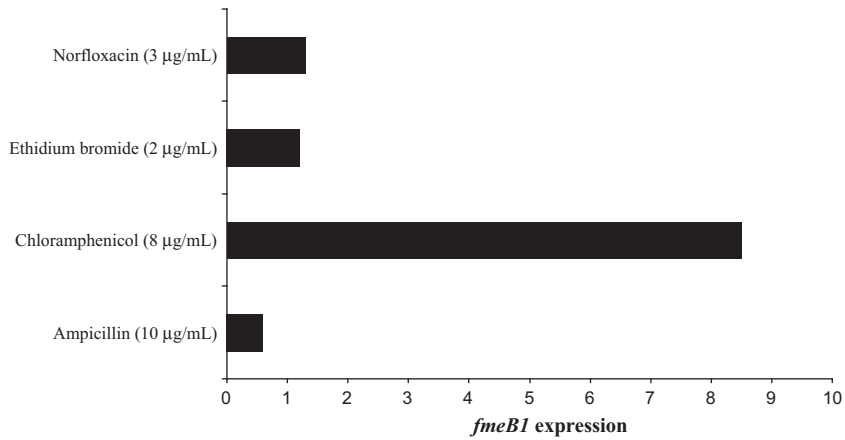
### 3.5. Chloramphenicol induction of *fmeB1* expression

qRT-PCR was used to measure relative expression of the five *mexB* homologs identified in *F. johnsoniae* G873 following exposure to sub-inhibitory concentrations of the following antimicrobial compounds and cytotoxic agents: chloramphenicol, norfloxacin, ampicillin, and ethidium bromide. It was found that exposure to 8  $\mu$ g/mL chloramphenicol resulted in an increase in the expression of the *mexB* homolog Fjoh\_4300, which we named *fmeB1*, in *F. johnsoniae* G873 (Fig. 1). This *mexB* homolog and the corresponding putative RND multidrug efflux pump system were hereby named FmeABC1. Exposure to ampicillin, norfloxacin, or ethidium bromide did not result in any significant change in *fmeB1* expression (Fig. 1). For all other *mexB* homologs in *F. johnsoniae* G873, there were no significant changes in expression due to exposure to any of the potential efflux pump substrates tested.

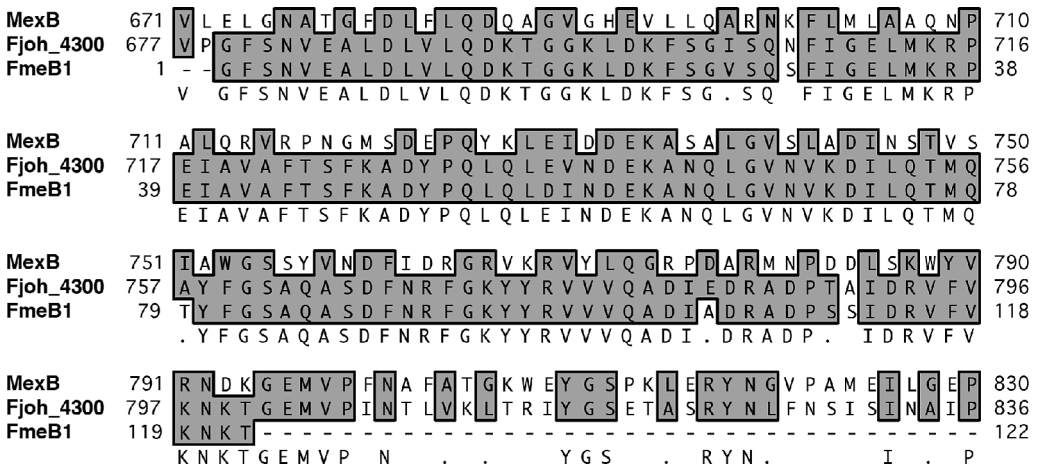
Following the qRT-PCR results, an approximately 350-bp partial sequence of the *fmeB1* gene was amplified in all seven *F. johnsoniae* isolates. A clustal alignment of the amino acid sequences of FmeB1, Fjoh\_4300, and MexB is shown in Figure 2. Partial sequences of the corresponding *mexA* homolog, *fmeA1*, were also amplified in all *F. johnsoniae* isolates (data not shown). The corresponding *oprM* homolog, *fmeC1*, was not successfully amplified in any of the *F. johnsoniae* isolates (data not shown).

### 3.6. Antibiotic MIC reduction with Pa $\beta$ N

The effect of the efflux pump inhibitor Pa $\beta$ N on the MIC values for a wide range of antibiotics was tested in *F. johnsoniae* G873 (Tab. I). A MIC decrease of four-fold or more in the



**Figure 1.** Semi-log bar graph illustrating the relative expression of *mexB* homolog Fjoh\_4300 hereafter named *fmeB1*. Quantitative real time RT-PCR compared expression of *fmeB1* in *F. johnsoniae* G873 with exposure to antibiotic compounds norfloxacin, chloramphenicol and ampicillin and cytotoxic compound ethidium bromide.



**Figure 2.** ClustalW alignment of amino acid sequences for MexB, (*P. aeruginosa* PA01), Fjoh\_4300, (*F. johnsoniae* 17061<sup>T</sup>), and FmeB1, (*F. johnsoniae* G873). Boxed, shaded residues indicate identical or similar amino acid sequences.

presence of PaβN was observed for chloramphenicol, florfenicol, norfloxacin, erythromycin, levofloxacin, linezolid, nitrofurantoin, and trimethoprim with sulfamethoxazole. These data provide phenotypic evidence for the presence of multiple substrates of efflux pumps in the *F. johnsoniae* isolates.

**4. DISCUSSION**

Classification of *Flavobacterium* species is complex, and often requires multiple identification methods for speciation of environmental isolates [5, 15]. The isolates used in this study were presumptively identified as *F. johnsoniae*

based on a combination of biochemical testing and 16S rRNA gene sequence determinations. These isolates were obtained from cultured brook trout. There have been relatively few studies linking *F. johnsoniae* with fish infectious disease [10, 15, 36], and the isolation of these *F. johnsoniae* isolates from clinical specimens represents additional evidence that this species is a putative opportunistic *Flavobacterium* pathogen.

Pathogenic *Flavobacterium* spp. represent a major threat to commercial and restoration aquaculture and the prevalence of MDR isolates demonstrates a growing need to advance our understanding of the antibiotic-resistance mechanisms of these bacteria [1, 9, 29]. The antibiotic-resistance determinants of *Flavobacterium*, as well as their close phylogenetic relatives *Chryseobacterium*, remain largely unresolved, likely due to the lack of available genetic tools [25, 42]. Of the  $\beta$ -lactamase genes that have been identified, many are distantly related to other  $\beta$ -lactamase genes or form new enzyme subclasses [4, 30]. Several of these  $\beta$ -lactamase genes were isolated by screening genomic libraries for antibiotic-resistant clones. Following initial unsuccessful attempts to identify antibiotic-resistance genes in the MDR *F. johnsoniae* isolates using primers from the literature for various classes of antibiotic-resistance determinants, genomic libraries of several strains were constructed (Tab. II). Screening of a genomic library made from isolate *F. johnsoniae* G873 resulted in the discovery of an amikacin-resistant clone containing a partial sequence of a gene encoding a TonB-dependent receptor protein. TonB enhances the resistance of the MexAB-OprM efflux system in *P. aeruginosa* [47]. Because of the previously established connection between TonB and RND multidrug efflux pumps, in addition to the failure to identify alternative antibiotic-resistance genes, the contribution of RND efflux pumps to the antibiotic resistance of the *F. johnsoniae* isolates was investigated. The presence of multidrug efflux pumps was also considered because RND multidrug efflux pumps are typically located on the bacterial chromosome, and plasmids were not detectable in these isolates.

Phenotypic evidence for the presence of functional RND multidrug efflux pumps was obtained using the efflux pump inhibitor Pa $\beta$ N. Pa $\beta$ N was originally identified in a screen for a broad-spectrum inhibitor of the *P. aeruginosa* Mex efflux pumps, and works as a competitive inhibitor of these and other similar RND multidrug efflux pumps [22, 28]. Pa $\beta$ N has been used in several investigations to screen for the presence of efflux pumps, often in clinical isolates [19, 24, 28]. In this study, the effect of Pa $\beta$ N on MIC values for a wide range of antimicrobial agents was tested in *F. johnsoniae* G873 (Tab. I). A four-fold or greater MIC decrease in the presence of Pa $\beta$ N was detected for a number of antibiotics, including chloramphenicol and florfenicol. The identification of florfenicol as a putative efflux pump substrate is important for aquaculture, because it is used for treatment of fish disease outbreaks [26]. These results reflect a similar reduction in florfenicol MIC values due to Pa $\beta$ N in *Chryseobacterium* [29], implying that efflux pump mediated florfenicol resistance is not exclusive to *F. johnsoniae*. Also, the Pa $\beta$ N reduction of chloramphenicol resistance complements the finding that chloramphenicol induces expression of *fmeB1* (Fig. 1). The remaining antimicrobials with decreased MIC values as a result of Pa $\beta$ N inhibition provide phenotypic evidence for the presence of multiple possible efflux pump substrates.

In conclusion, this study represents the identification and initial characterization of an RND multidrug efflux pump system, hereby named FmeABC1, in *F. johnsoniae*. This nomenclature follows the convention established by Ueda et al. for newly identified Mex efflux pump system homologs in *Bacteroides* [40]. The expression of *fmeB1*, 1 of 5 *mexB* homologs identified in *F. johnsoniae* G873, was induced by exposure to chloramphenicol. These data demonstrate that there may be multiple efflux pump substrates in specific strains of *F. johnsoniae*. The contribution of efflux pump mediated antibiotic resistances should be factored into decisions regarding the efficacy of clinical treatments of fishes associated with flavobacteria epizootics.



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