

## Iron Shortage and Bile Salts Play a Major Role in the Expression of *ompK* Gene in *Vibrio anguillarum*

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### Abstract

The outer membrane protein K, OmpK first identified in *Vibrio parahaemolyticus* has been shown to be a receptor for a broad host range vibriophage KVP40 infecting members of the *Vibrionaceae*. In the study, the effect of culture conditions on the expression of *ompK* in *V. anguillarum* was studied using real-time PCR. The expression increased significantly in the presence of bile salts and iron chelating agent 2, 2' bipyridine, suggesting a role for this protein in bile resistance and also in iron acquisition by *V. anguillarum*. OmpK induction by iron limitation and the presence of bile salts was reconfirmed by western blot technique after growing the cells in trypticase soy broth supplemented with bile salts, blood and 2, 2' bipyridine. We surmise that the expression of OmpK protein of *V. anguillarum* is bile salt and iron chelating agent-dependent.

**Key words:** *Vibrio anguillarum*, OmpK expression, Real-time PCR, Western blot, bile salt, iron deprivation

### Introduction

*Vibrio anguillarum*, a marine bacterium belonging to the family *Vibrionaceae* is a major bacterial pathogen of marine and estuarine fish throughout the world (Myhr *et al.*, 1991). *V. anguillarum* infection results in fatal haemorrhagic septicaemia in marine fish when the immune system of fish is compromised or when the mucosal surfaces are damaged (Croxatto *et al.*, 2007).

The outer membrane of Gram-negative bacteria consists of phospholipids and proteins. The proteins make up to 50% of the outer membrane mass. In recent years, these outer membrane proteins have attracted attention, since they have an important role in the host-bacteria interaction in adherence, uptake of nutrients including iron from the host, and subverting host defense mechanisms (Li *et al.*, 2008).

To date, studies on *V. anguillarum* outer membrane proteins have focused on OmpU, the major outer membrane protein present in large quantities and OM2, the induced outer membrane protein, which is known to be linked with virulence of the organism (Pedersen *et al.*, 1997; Simón *et al.*, 1996; Wang *et al.*, 2003). However, more interest has been given to the role of membrane proteins involved in iron uptake such as FetA and FvtA.

Recently it was demonstrated that the expression of these proteins decreases spectacularly with iron readiness (Naka and Crosa, 2012). Some Omps play a role in efflux of salinity, therefore their expression increases with the rise of salt concentration (Davey *et al.*, 1998, Kao *et al.*, 2009).

Although the presence of OmpK has been demonstrated in *V. cholerae*, *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus* (Inoue *et al.*, 1995a), only the protective efficacy of *V. alginolyticus* OmpK against fish vibriosis caused by *V. alginolyticus* has been reported (Qian *et al.*, 2008). Therefore in this study, we attempted to look for OmpK at DNA and protein level; in addition, we also studied the expression of *ompK* gene by real-time PCR and the effect of different culture conditions on the expression to get an insight on the role of this outer membrane protein in *V. anguillarum*.

### Experimental

#### Materials and Methods

**Bacterial strain and growth condition.** *V. anguillarum* NB10 kindly provided by Dr. Sahul Hameed (Abdul Hakeem College, India) was maintained at

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-80°C in nutrient broth containing 30% glycerol (Sanyo Corporation, Japan). The culture was set up by inoculating 5 ml of sterile trypticase soya broth (TSB) supplemented with 1% NaCl and incubated overnight at 28°C.

**Nucleic acid extractions.** DNA extraction was carried out from 1 ml of *V. anguillarum* culture using the method described by Ausubel *et al.*, 1995. The DNA pellet was resuspended in Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). RNA extraction and purification was done by RNeasy Protect Bacteria Mini Kit (Qiagen, USA) according to the protocol described by the manufacturer. Extracts were subsequently treated with DNase I (Fermentas International Inc., Canada), according to manufacturer's guidelines to remove any remaining DNA.

**Primer design.** To study the *ompK* gene of *V. anguillarum*, the primers F-k502: GGCTTCCAAATTTCAACCAA and R-k699: TTTAAGGCCGTAGCCAACAG were designed using the Primer3 software ([http://biotools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi)), based on the *ompK* gene (GenBank accession no. FJ705222.1). The primers F-*rpoA*: CGTAGCTGAAGGCAAAGATGA and R-*rpoA*: AGCTGGAACATAAC-CACGA previously reported by Defoirdt *et al.*, 2007 were used to amplify *rpoA*, which served as an internal standard gene.

**Effect of growth conditions.** The experiment was carried out in 100 ml TSB inoculated with 1 ml overnight culture followed by incubation at 28°C. To study the effect of NaCl on *V. anguillarum* growth, bacteria were grown in TSB supplemented with the following NaCl concentrations; 1% (control), 2%, 3% and 4%. To study the effect of bile salts, the culture was grown in media containing 0.1%, 0.2%, and 0.4% bile salts, and also grown in media free of bile salt (control). The effect of blood was determined by using 1%, and 2% of fresh blood; culture grown in media without blood were taken as control. The effect of the iron chelating agent 2, 2'-bipyridine was studied by growing the bacteria with or without 20 mM 2, 2'-bipyridine. The effect of a combination of 1% blood and 100 mM of 2, 2'-bipyridine was also studied.

**cDNA synthesis.** Reverse transcription was carried out according to the protocol of Fermentas Life Sciences (Fermentas International Inc., Canada). Briefly, the RNA was reverse transcribed to cDNA from 2 µg of RNA using 2 µl of reverse primer (100 ng/µl) and 0.5 µl of RevertAid H minus (Fermentas International Inc., Canada) at 42°C for 1 h. *OmpK* cDNA was tested by PCR using primers F-k502, and R-k699 and cDNA of the internal standard gene (*rpoA* subunit) was tested by PCR using primers F-*rpoA* and R-*rpoA*. cDNA samples were stored at -20°C for further use.

**Real-time PCR analysis.** Quantification of expression level of the *ompK* gene was done by real-time PCR

using 7300 Real-time PCR System (Applied Biosystems, Foster city, USA). Validation of the real-time PCR was done by amplifying serial dilutions of cDNA synthesized from 1 µg of RNA isolated from bacterial samples (Livak and Schmittgen, 2001). to carry out relative quantification using the  $2^{-\Delta\Delta Ct}$  formula.

The appropriate primer concentrations (200 nM for *ompK* and 300 nM for *rpoA*) were used for thermal cycling which included an initial activation at 50°C for 2 min, initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 60°C for 45 s and elongation at 72°C for 30 s and a melting-curve program (95°C for 15 s and 60°C for 30 s). This was performed for each gene to check for amplification of the targeted fragments. Each reaction was performed in triplicate and the average value was considered for data analysis and statistical validation. Amplification data was recorded at the end of each elongation step.

Analysis of relative gene expression was performed using the  $2^{-\Delta\Delta Ct}$  method of Livak and Schmittgen (2001). The expression of the target genes was normalized to the endogenous control by calculating  $\Delta C_T$  and expressed relative to a calibrator by calculating  $\Delta\Delta C_T$  ( $\Delta\Delta C_T = \Delta C_T^{\text{target}} - \Delta C_T^{\text{calibrator}}$ ). The control values (5 h time interval, 0.5% NaCl, 0% bile salt, iron and 2, 2'-bipyridine) of each experimental condition were used as calibrator. The expression level of the *ompK* gene in each condition of the study was tested by an independent sample student t-test for calculating  $\Delta Ct$  data. Significance level was taken at 5%.

**Western blotting.** Bacteria grown under different culture conditions that resulted in a significant difference in the production of *ompK* gene were harvested by centrifugation at 4000 g for 10 min and were lysed by discontinuous sonication on ice at 20–30 W with six 10 s bursts followed by centrifugation at 5000 g for 20 min to remove the unlysed cells. Cell lysates were resolved in 15% SDS-PAGE (without staining) and electrotransferred onto nitrocellulose blotting membrane (Pall, New York, USA) at a constant voltage of 50 V for 1 h according to our previous article (Hamod *et al.*, 2012). The membrane was blocked with 3% bovine serum albumin (BSA) overnight at 4°C. This was followed by three times washes with phosphate buffer saline containing 0.05% Tween-20 (PBST) followed by 3 washes with phosphate buffer saline (PBS) (1 min each time). The protein bands on the membrane were allowed to react with the rabbit anti-OmpK serum developed by us as described in our previous work (Hamod *et al.*, 2012) diluted 1:100 with gentle agitation at room temperature for 1 h, followed by washing and further incubation with horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibodies (Sigma, Missouri, USA) diluted 1:1000 for

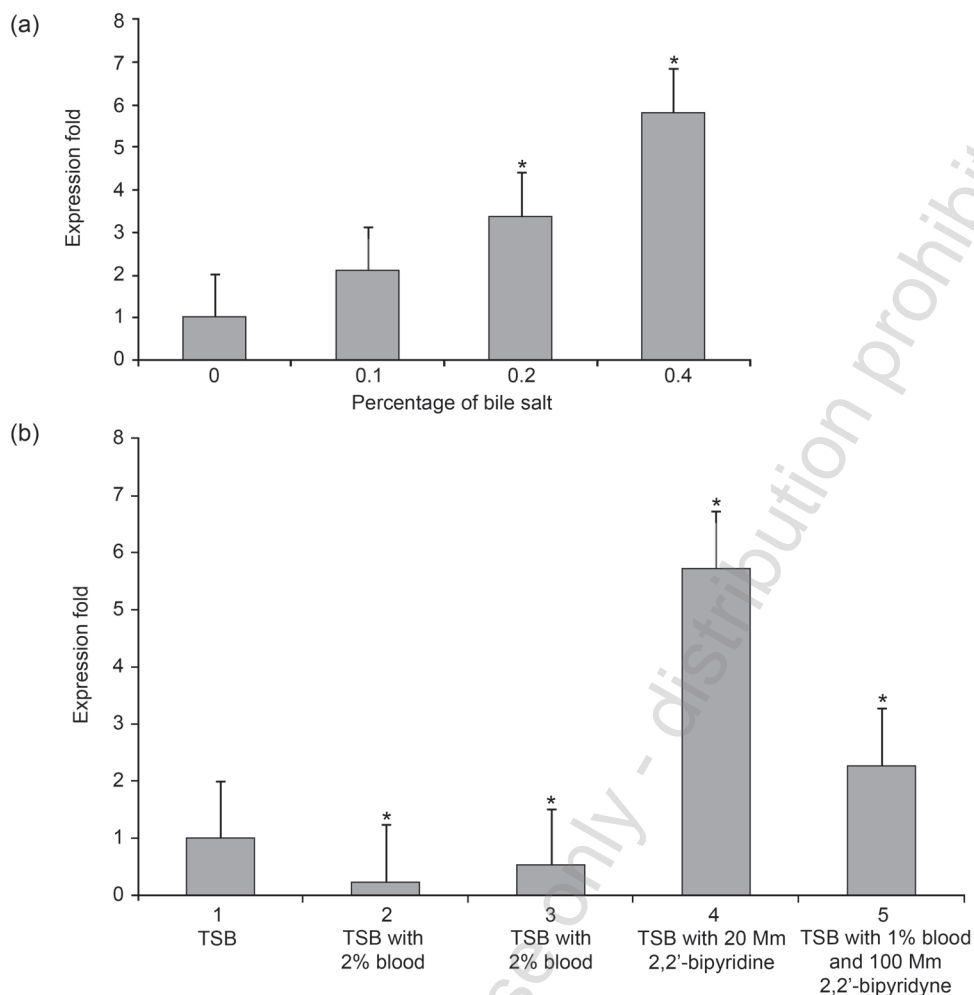


Fig 1. Expression of *V. anguillarum ompK* gene (a) in the presence of different concentrations of bile salts (b) in the presence of 1% blood, 2% blood, 20 Mm 2, 2'-bipyridine and 1% blood with 100 Mm 2, 2'-bipyridine. The error bars represent the standard deviation of three independent measurements of  $\Delta\Delta CT$  based on bacterial mRNAs. Data that are significantly different ( $P < 0.01$ ) from the calibrator are marked with an asterisk.

1 h. The reaction was carried out by dipping the membrane in tetramethylbenzidine solution (Merck, India) after thorough washing with PBS, stopped by rinsing with distilled water and visualized.

## Results

**Effect of culture conditions on the expression of *ompK* gene.** Study on the effect of varying salt concentrations on growth and *OmpK* expression of *V. anguillarum* NB10 showed that NaCl concentrations up to 3% had a minimal effect. Expression of *ompK* at 2% NaCl (2.74 fold) was observed to be higher than at 1%, 3% and 4% of NaCl. The growth of *V. anguillarum* NB10 seemed to decrease with increasing bile salt concentration in the medium while the expression of *ompK* increased with increase in bile salt concentration (Fig. 1a). Expression of *ompK* gene was the highest at 0.4% bile salt concentration (5.8 fold) (Fig. 1a). Supplementation of iron chelating agent 2, 2'-bipyridine

increased *ompK* 5.7 fold (Fig. 1b). On supplementation of culture medium with fresh human blood, the expression of *ompK* decreased (Fig. 1b). When both 20 mM 2, 2'-bipyridine and blood were supplemented, *ompK* expression was reduced to 2.3 fold compared to 5.7 fold in the presence of 2, 2'-bipyridine alone (Fig. 1b).

**Western blot analysis.** Positive bands with molecular weight of 28 KDa were seen only in the lanes containing cell lysates of *V. anguillarum* grown with 0.4% of bile salts, 20 mM bipyridine, and 1% of blood with 100 mM bipyridine (Fig. 2). No bands appeared in the other lanes containing the cell lysates of *V. anguillarum* grown with 4% NaCl, 2% blood supplement or bacteria grown in TSB without any supplementation.

## Discussion

*V. anguillarum* is a causative agent of vibriosis in finfish and invertebrates such as bivalves and crustaceans. Vibriosis results in massive losses to the aquaculture

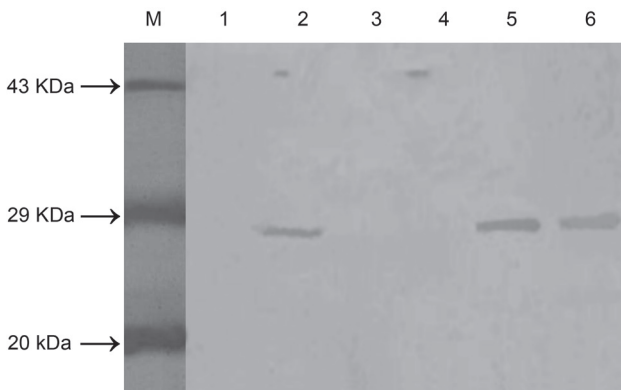


Fig. 2. Western blot for *V. anguillarum* OmpK grown in presence of bile salts, NaCl, bipyridine and blood. Protein marker (M), TSB (1), 0.4% bile salt (2), 4% NaCl (3), 2% of blood (4), 20 mM bipyridine (5), 1% of blood and 100 mM bipyridine(6).

industry (Garcia *et al.*, 1997). *V. anguillarum* NB10 initially isolated by Norqvist *et al.*, 1989 was the representative isolate chosen for this study, since it belongs to serotype O1, which is globally accepted as a highly pathogenic serotype. Since serotype O1 assumes variable outer membrane profile under different conditions (Simón *et al.*, 1996), the use of a strain of this serotype would be appropriate for the study of any novel outer membrane protein.

Gram-negative bacteria such as *V. anguillarum* are surrounded by a bilayer outer membrane in which proteins are interspersed. Porins in bacterial outer membranes have a regulatory role in nutrient uptake, facilitating the transport of specific nutrient molecules (Achouak *et al.*, 2001; Nikaido, 1994). Few outer membrane proteins such as OmpA and general porins are constitutively expressed in high levels in the cell whereas the majority of outer membrane proteins are induced with increased synthesis occurring only under certain circumstances (Koebnik *et al.*, 2000). A 26 kDa outer membrane protein, OmpK common to *Vibrio* species was first reported by Inoue *et al.* (1995a; 1995b) and shown to serve as a receptor for a broad-host-range vibriophage, KVP40. Although the genes encoding OmpK in *V. cholerae*, *V. harveyi* and *V. alginolyticus* are similar (Inoue *et al.*, 1995a), variations for this gene in terms of the number of amino acids they encode have been reported for *V. alginolyticus* and *V. harveyi* (Li *et al.*, 2008; Qian *et al.*, 2008). So far, there has been no report on the structure and functions of *ompK* gene in *V. anguillarum*.

Polyclonal antibody was raised against the purified recombinant OmpK protein in rabbit in our work (Hamod *et al.*, 2012). Surprisingly a positive band was detected only in the case of purified OmpK protein. The protein in the cell lysate of *V. anguillarum* was undetected by Western blot. It has been reported that expression of OMP is related to environmental condi-

tions like temperature, salinity, availability of nutrients and oxygen (Nandi *et al.*, 2005).

Real-time PCR technique, a reliable quantitative method for measuring gene expression (Nielsen and Boye, 2005) was used in this study to determine the effect of varying environmental factors such as salt concentration, bile salt concentration and iron chelating agent on the expression of *ompK* gene in *V. anguillarum*.

*V. anguillarum*, a marine bacterium, well recognized as a pathogen of marine and estuarine fish, encounters different osmotic environments. The outer membrane proteins have a crucial role in maintaining the osmotic state of the organism during any salt stress. In this study growing *V. anguillarum* at salt concentrations higher than 2%, which is the optimum for its growth, did not upregulate *ompK* expression suggesting that *ompK* probably is not involved in osmoregulation. On the contrary, using the proteomic approach of Kao *et al.*, 2009 showed that both *ompU* and *ompW* are highly expressed at 3.5% NaCl and are possibly involved in osmoregulatory function in *V. anguillarum*.

*V. anguillarum* being a fish pathogen, when ingested by fish is likely to be exposed to bile in the fish gut and therefore might be in possession of mechanisms for survival in the gut environment. Our study shows that expression of *ompK* is upregulated in the presence of bile salts. Though growth seemed to be suppressed at 0.4% bile salt (data not shown), expression of *ompK* was 5 fold higher (Fig. 1a) suggesting a role for this protein in bile resistance. Wang *et al.*, 2003 showed OmpU to play a role in bile resistance in *V. anguillarum*. In *Escherichia coli* two outer membrane proteins OmpF and OmpC confer bile salt resistance. While OmpF is sensitive to bile salt concentration changes at low temperature and salinity, OmpC responds to alteration in bile salt concentration at elevated temperature (Nikaido and Rosenberg, 1983). Similarly, we suggest that in *V. anguillarum*, in addition to OmpU (Wang *et al.*, 2003), OmpK may also be playing an important role in bile salt resistance and its uptake.

Iron uptake mechanisms are involved in conferring virulence to *V. anguillarum*. Two iron uptake mechanisms have been reported in this organism viz production of anguibactin and vanchrobactin (Lemos *et al.*, 1988). We observed that expression of *ompK* was induced in the presence of iron chelating agent 2,2'-bipyridine and this is partially offset if blood is present in the medium (Fig. 1b), which probably suggests that OmpK in *V. anguillarum* responds to changes in iron level in the environment.

Therefore, *V. anguillarum* strain was grown in TSB medium supplemented with 20 m mol<sup>-1</sup> 2,2'-bipyridine, 0.4% bile salt, 4% NaCl, 2% of blood and combination of bipyridine and blood for the detection of OmpK protein by Western blot. A band with 28 kDa (Fig. 2) was

visualized in the case of *V. anguillarum* strain grown in bile salt, bipyridine and combination of blood and bipyridine. This result further confirms the role of OmpK protein in bile salt resistant and iron uptake.

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