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ORIGINAL PAPER

Indole contributes to tetracycline resistance via the outer membrane protein OmpN in *Vibrio splendidus*

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Abstract

As an interspecies and interkingdom signaling molecule, indole has recently received attention for its diverse effects on the physiology of both bacteria and hosts. In this study, indole increased the tetracycline resistance of *Vibrio splendidus*. The minimal inhibitory concentration of tetracycline was 10 μg/mL, and the OD_{600} of *V. splendidus* decreased by 94.5% in the presence of 20 μg/mL tetracycline; however, the OD_{600} of *V. splendidus* with a mixture of 20 μg/mL tetracycline and 125 μM indole was 10- or 4.5-fold higher than that with only 20 μg/mL tetracycline at different time points. The percentage of cells resistant to 10 μg/mL tetracycline was 600-fold higher in the culture with an OD_{600} of approximately 2.0 (higher level of indole) than that in the culture with an OD_{600} of 0.5, which also meant that the level of indole was correlated to the tetracycline resistance of *V. splendidus*. Furthermore, one differentially expressed protein, which was identified as the outer membrane porin OmpN using SDS-PAGE combined with MALDI-TOF/TOF MS, was upregulated. Consequently, the expression of the *ompN* gene in the presence of either tetracycline or indole and simultaneously in the presence of indole and tetracycline was upregulated by 1.8-, 2.54-, and 6.01-fold, respectively, compared to the control samples. The combined results demonstrated that indole enhanced the tetracycline resistance of *V. splendidus*, and this resistance was probably due to upregulation of the outer membrane porin OmpN.

Keywords *Vibrio splendidus* · Indole · Tetracycline resistance · Outer membrane protein

Introduction

Apostichopus japonicus is an economically important aquaculture species in China (Han et al. 2016), but this organism can be infected by many diseases, due to its high density during culture. Skin ulcer syndrome (SUS) is one of the most serious diseases of *A. japonicus*, resulting in high mortalities of 90% to 100% and great economic losses (Wang et al. 2005a; Zhang et al. 2006; Deng et al. 2009). Antibiotics are effective agents that have been frequently used to protect *A. japonicus* from pathogen infections (Wang et al. 2005b; Han et al. 2016). For example, oxytetracycline was effective in combating the SUS of *A. japonicus* caused by bacteria in Shandong Province, China (Wang et al. 2005a, b). Tetracycline has good oral absorption, causes few allergic reactions and is relatively inexpensive, making it a nearly perfect therapeutic agent; therefore, it has been used intensively in the control of bacterial infections in both humans and animals over the past years (Schnappinger and Hillen 1996). However, bacteria begin to show resistance to antibiotics within as little as a few years after the first use and multidrug resistant bacteria have now developed and have been regarded as one of the most important threats to public health (Hall 2004; Hancock 2014; Page and Bush 2014).

Outer membrane proteins (OMPs) act as a permeability barrier that protects the cell from extracellular stresses, such as antimicrobial agents in Gram-negative bacteria (Xu et al. 2006). Antibiotic susceptible bacteria can acquire antimicrobial resistance (AMR) by the activation of OMPs (Urfer

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et al. 2015), which is important for the survival of Gram-negative bacteria under antibiotic stress (Weatherspoon-Griffin et al. 2014). For example, bacteria acquired AMR by upregulating the expression of genes involved in multidrug efflux pumps, such as MdtAE, CusB, EmrK, and YceL (Nikaido et al. 2012), the two-component regulatory system CpxAR in *Escherichia coli* (Hirakawa et al. 2005), AcrAB in *Salmonella enterica* (Nikaido et al. 2012), TtgGHI in *P. putida* (Molina-Santiago et al. 2014), and EmrA, NorM and Atu25521 in *Agrobacterium tumefaciens* (Lee et al. 2015). Indole is ubiquitously produced by bacteria and has attracted great attention because of its regulation on bacterial physiology, including AMR (Hirakawa et al. 2005; Lee et al. 2010a, b; Vega et al. 2012, 2013). For example, *Pseudomonas aeruginosa* and *Pseudomonas putida* acquired ampicillin resistance after growing on agar containing indole/ampicillin (Kim et al. 2017).

Vibrio splendidus has been identified as one of the most important opportunistic pathogens that can infect *A. japonicus* (Zhang et al. 2006), bivalves (Sugumar et al. 1998; Ben Cheikh et al. 2017) and different kinds of fish (Gatesoupe et al. 1999; Macpherson et al. 2012). One of its OMPs, OmpU, contributes to resistance to antimicrobial peptides and is required for virulence of the oyster *Crassostrea gigas* (Duperthuy et al. 2010). But until now, no other OMPs have been identified in *V. splendidus*. In the present study, the effects of tetracycline and indole, individually and combined on the growth of *V. splendidus* were determined. Cells were cultured in the presence of indole, and differentially expressed proteins were identified using SDS-PAGE combined with MALDI-TOF/TOF MS. Furthermore, differential expression of the *ompN* gene in the presence of tetracycline, indole, and both indole and tetracycline was also determined using real-time reverse transcriptase PCR (RT-PCR). Finally, a preliminary mechanism was proposed to illustrate the phenomenon that indole increased tetracycline resistance in *V. Splendidus.*

Materials and methods

Bacterial strains, culture conditions and chemicals

Vibrio splendidus was cultured in 2216E medium consisting of 5 g/L tryptone, 1 g/L yeast extract, and 0.01 g/L $FePO_4$ in aged seawater at 28 °C. Indole was dissolved in ethanol to make a stock solution of 1 M. Unless otherwise stated, all other chemicals used in this study were purchased from Sangon (Shanghai, China).

Determination of minimal inhibitory concentration (MIC)

The MIC was determined according to the study of Wiegand et al. (2008) with minor modifications. Briefly, overnight cultures were collected and washed twice with PBS. Approximately 1.0×10^6 CFU/mL cells were inoculated into fresh 2216E medium supplemented with various concentrations of tetracycline. The concentrations of tetracycline were 0, 2.5, 5, 7.5, 10, 12.5, 15, and 20 μg/mL. Then, all samples were cultured at 28 °C for 24 h, and aliquots were taken for OD_{600} measurements using a UV-1600PC instrument (MAPADA, China). The MIC was defined as the minimal antibiotic concentration that almost inhibited growth after culture for 24 h.

Growth measurement

Vibrio splendidus was cultured in 2216E medium to an OD_{600} of approximately 1.0, diluted 10^3-fold using fresh medium and incubated for 1 h. Then, the cells were divided into four groups. One group was supplemented with 125 μM indole, the second group was supplemented with 20 μg/mL tetracycline, the third group was simultaneously supplemented with 20 μg/mL tetracycline and 125 μM indole, and the last group was supplemented with ethanol as a control. Then, the cultures were grown for an additional 24 h, and aliquots were taken for OD_{600} measurements using a UV-1600PC instrument (MAPADA, China).

Tetracycline resistance

Vibrio splendidus was cultured in 2216E medium overnight to an OD_{600} of approximately 2.0, and the cells were inoculated into fresh 2216E medium at a volume of 1% (v/v). The cells were cultured until the OD_{600} reached 0.5. Then, the overnight culture (OD_{600} of 2.0) and the exponential culture (OD_{600} of 0.5) were serially diluted tenfold, and a 50 μL aliquot was plated onto 2216E agar. For antibiotic treatment, both kinds of cells were resuspended in PBS and then treated with 10 μg/mL tetracycline for 3 h. Then, the cells were serially diluted tenfold, and a 50 μL aliquot was plated onto 2216E agar. All plates were incubated at 28 °C for 16 h, followed by colony counting.

Identification of differentially expressed proteins by SDS-PAGE combined with MALDI-TOF/TOF MS

Vibrio splendidus was cultured in 2216E medium to an OD_{600} of approximately 0.2, then bacteria were reinoculated into fresh medium supplemented with 20, 50, 100, and 125 μM indole. *V. splendidus* cultured with ethanol was used

as a negative control. Following growth for an additional 2 h, cultures were centrifuged at 8000×*g* for 10 min. The supernatant was removed, and the cells were suspended in cell lysis buffer containing 8 M urea. The distinct differentially expressed bands among the samples were collected after SDS-PAGE and subjected to MALDI-TOF/TOF MS for protein identification as described previously (Crestani et al. 2012). MS was performed on an ABI 5800 MALDI-TOF/TOF MS instrument (Applied Biosystems, Foster City, USA). Data were acquired through a positive MS reflector by using a CalMix5 standard for calibration of the instrument (ABI5800 calibration mixture).

Sample collection for real-time RT-PCR

Vibrio splendidus was cultured in 2216E medium to an OD_{600} of approximately 0.2, when the cells did not produce indole (Zhang et al. 2017). *V. splendidus* was then continuously cultured with 125 μM or without indole for 10 min, 30 min and 60 min. Cells were collected and RNA was extracted for real-time RT-PCR to determine the effects of indole on the differential protein expression. To detect the effects of tetracycline and the mixture of tetracycline and indole on gene expression, *V. splendidus* was grown in 2216E medium to an OD_{600} of approximately 0.2, and then the culture was reinoculated into fresh 2216E medium supplemented with 20 μg/mL tetracycline or 20 μg/mL tetracycline and 125 μM indole. *V. splendidus* cultured in 2216E with ethanol was used as a control. All samples were cultured for another 30 min, and then cells were collected by centrifugation, and RNA was extracted to be further used for real-time RT-PCR.

Real-time RT-PCR

Real-time RT-PCR was carried out as described by Zhang et al. (2013). Total RNA was extracted using the bacterial RNA kit (Omega). cDNA was synthesized from 250 ng of total RNA by a Reverse Transcription System using random hexamers according to the regent's instructions (Takara, China). Real-time RT-PCR was carried out in an ABI 7500 real time detection system by using the Sybr ExScript RT-PCR kit (Takara, China). The primers for the expression of the differentially expressed protein were ompNRTF (5′-CGCTATCGACTTCGGAAACG-3′) and ompNRTR (5′-AACACCCGCAAGGTACAGACC-3′). Each assay was performed in triplicate using 16S rDNA as a reference gene. The primers used for 16S rDNA were 933F (5′-GCACAAGCGGTGGAGCATGTGG-3′) and 16SRTR1 (5′-CGTGTGTAGCCCTGGTCGTA-3′). The comparative threshold cycle method $2^{-\Delta\Delta CT}$ was used to analyze the relative mRNA level of the target gene.

Results

Indole increased the tetracycline resistance of *V. splendidus*

The MIC of tetracycline to *V. splendidus* was 10 μg/mL in this study (Fig. 1). The OD_{600} of *V. splendidus* decreased linearly with an increase in tetracycline from 0 to 10 μg/mL. Almost no growth was observed when the concentration of tetracycline was higher than 10 μg/mL. The OD_{600} in the presence of 20 μg/mL tetracycline was 5.5% of that without tetracycline added. In addition, the 20 μg/mL tetracycline had the most stable inhibitory effect under an extended culture time (data not shown). Therefore, 20 μg/mL tetracycline was chosen to examine the effect of exogenous indole on *V. splendidus* under antibiotic stress. The concentration of indole used was 125 μM, which was the level produced during early stationary phase and showed no effect on the growth of *V. splendidus* (Fig. 2). After grown for 24 h, the OD_{600} of the culture supplemented with 125 μM indole increased 4.5-fold compared with that of the culture supplemented with 20 μg/mL tetracycline only (Fig. 2). These results demonstrated that tetracycline significantly inhibited the growth of *V. splendidus*, while indole alleviated the stress created by tetracycline. In our previous study (Zhang et al. 2017), indole accumulated in the culture supernatant during the growth of *V. splendidus*. After *V. splendidus* was grown for 24 h, the level of indole reached approximately 150 μM. In the present study, both exponential and stationary cells were collected, washed and resuspended in PBS containing 10 μg/mL tetracycline for 3 h. The percentage of surviving cells in the culture with an OD_{600} of 2.0 was 600-fold higher than that in the culture with an OD_{600} of 0.5 (Fig. 3). All of the above

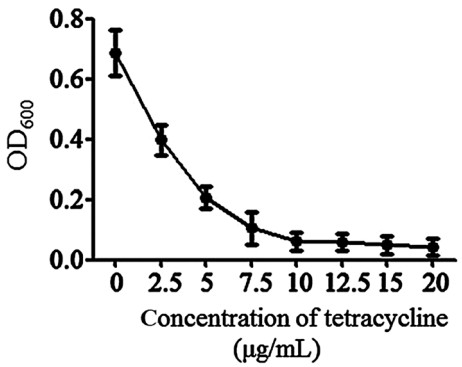

Fig. 1 Growth of *V. splendidus* in 2216E medium supplemented with different concentrations of tetracycline. Aliquots were taken for OD_{600} measurements after culture for 24 h. The data are the means for at least three independent experiments and are presented as the means ± SD

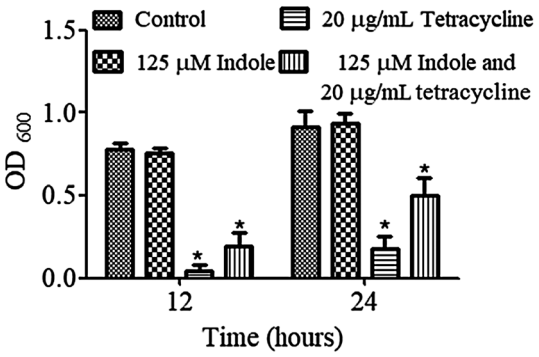

Fig. 2 Effects of 20 μg/mL tetracycline, 125 μM indole and a mixture of 20 μg/mL indole and 125 μM tetracycline on growth of *V. splendidus*. After the cells grew to an OD_{600} of approximately 1.0, the cells were incubated in medium supplemented with indole, tetracycline, or tetracycline with indole. *V. splendidus* cultured with ethanol was used as a control. Aliquots were taken at different time points for OD_{600} measurements. The data are the means for at least three independent experiments and are presented as the means ± SD

results suggested that indole significantly increased the tetracycline resistance of *V. splendidus*.

Identification of differentially expressed proteins

The differentially expressed proteins of *V. splendidus* grown in the presence or absence of indole were determined using SDS-PAGE. One distinctive band with an approximate molecular weight of less than 40 kDa was detected (Fig. 4a). Subsequently, the differentially expressed protein was identified using MS, and the result indicated that it was the outer membrane protein OmpN (Fig. 4b). Based on our sequence data and BLAST results, the differentially expressed protein was identified as a 345 amino acid outer membrane protein, the sequence of which is shown in Fig. 5a, encoded by a 1083 bp gene. The nucleotide sequence showed similarities of 77.12% and 76.47% to the OmpNs from *Vibrio vulnificus* (CP019291.1) and *V. splendidus* LGP32 (FM954973.2), respectively. SMART analysis showed that OmpN contained an obvious signal peptide from 1 to 21 aa (Fig. 5a, b). SWISS-MODEL analysis

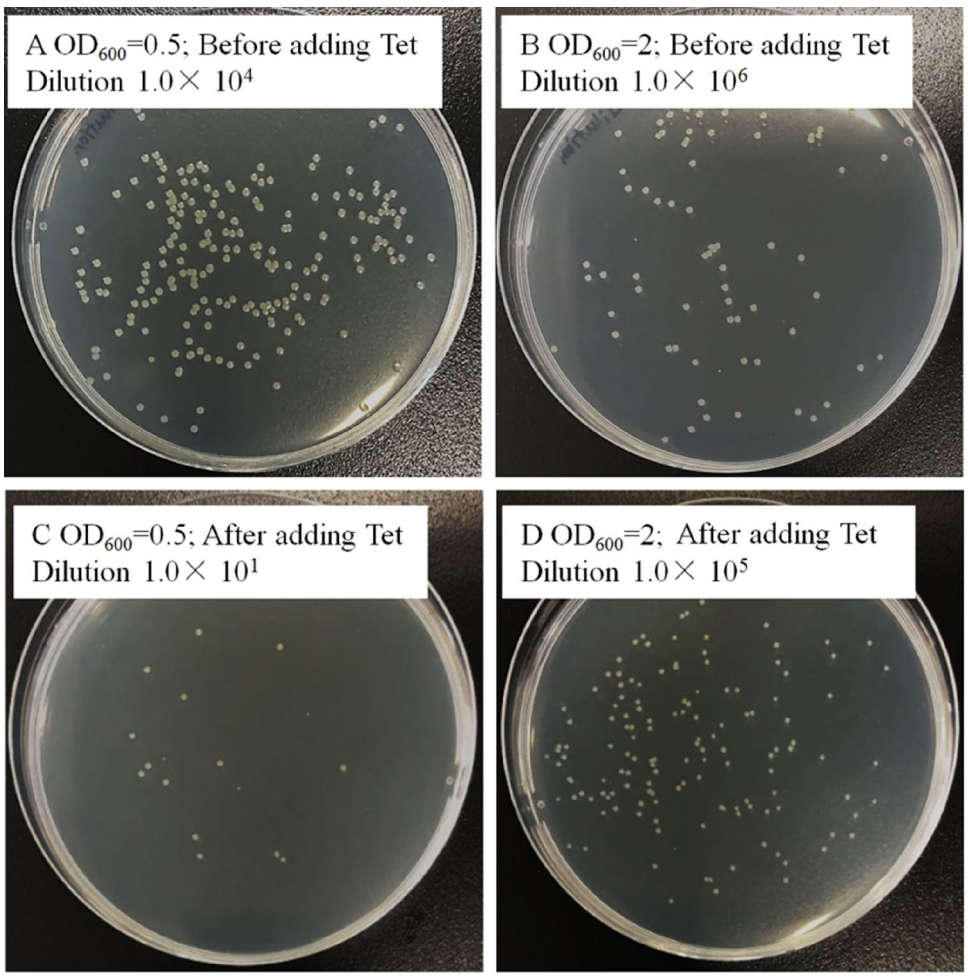

Fig. 3 Colony counting of cultures with different OD_{600} values before and after tetracycline treatment. **a** Cells with an OD_{600} of 0.5, 1.0×10^4-fold dilution; **b** cells with an OD_{600} of 2.0, 1.0×10^6-fold dilution; **c** tetracycline-treated cells with an OD_{600} of 0.5, tenfold dilution; **d** tetracycline-treated cells with an OD_{600} of 2.0, 1.0×10^5-fold dilution. Fifty-microliter aliquots of each dilution were plated on 2216E agar and incubated at 28 °C for 24 h. Then the colonies were counted

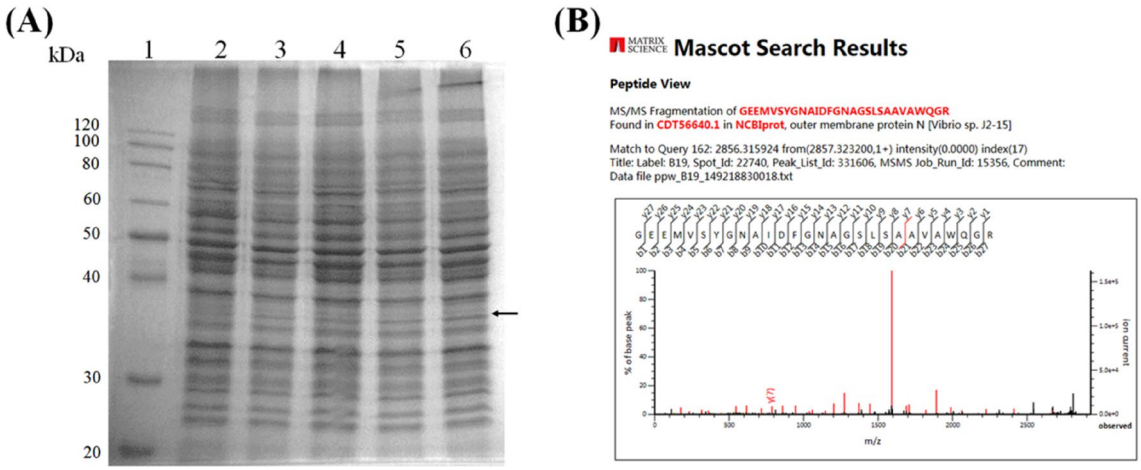

Fig. 4 **a** Identification of upregulated protein. SDS-PAGE profiles of *V. splendidus* in the absence and presence of different concentrations of indole. Lane 1, protein marker; lane 2, protein sample from *V. splendidus* grown without indole; lanes 3–6, protein samples from *V. splendidus* in the presence of 20, 50, 100, and 125 μM indole. The distinct differentially expressed protein is marked with an arrow. **b** One of the peptides identified using MS

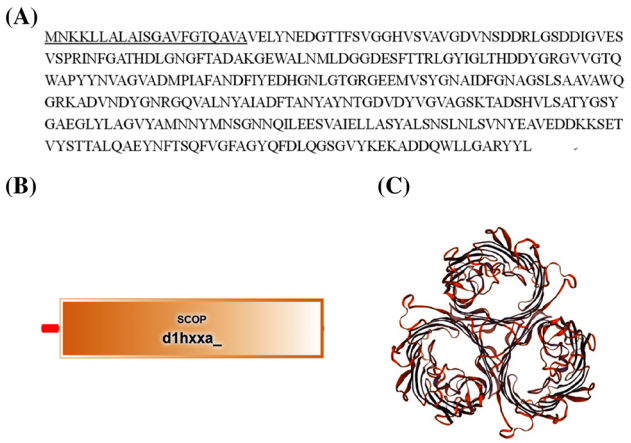

Fig. 5 **a** Amino acid sequences of the outer membrane protein OmpN from *V. splendidus*; **b** The typical domain of OmpN predicted using SMART; **c** The structure of the OmpN predicted using SWISS-MODEL

showed that OmpN is a homologous trimer that possessed three parallel β-barrel structures similar to a porin (Fig. 5c).

Indole and tetracycline increased the gene expression of *ompN*

To further explore the effect of indole on the expression of the *ompN* gene, *V. splendidus* was grown to an initial OD_{600} of 0.2, and then the culture was divided into two groups. One group was supplemented with 125 μM indole, and the other group was supplemented with ethanol as a control. Following culture for 10 min, 30 min and 60 min, the mRNA levels of the *ompN* gene in *V. splendidus* grown with 125 μM indole were first upregulated to 2.54- and 1.5-fold compared with the control, and then recovered to the normal level at 60 min (Fig. 6a). This result indicated that indole increased expression of the *ompN* gene in *V. splendidus* at mRNA level. To investigate the effects of tetracycline on the expression of the *ompN* gene, tetracycline was added to the *V. splendidus* culture at an OD_{600} of 0.2. After culturing for 30 min, the expression of the *ompN* gene was upregulated by 1.8-fold compared with the expression in *V. splendidus* cultured without tetracycline. In the simultaneous presence of indole and tetracycline, the expression of the *ompN* gene was significantly upregulated by 6.01-fold compared with the expression in cells supplemented with ethanol (Fig. 6b). These results suggest that OmpN is probably responsible for tetracycline resistance in *V. splendidus* and that indole increased tetracycline resistance by stimulating the expression of the *ompN* gene.

Discussion

In previous reports, indole was found to increase drug resistance in *Salmonella enterica* (Nikaido et al. 2012; Vega et al. 2013), *E. coli* (Vega et al. 2013) and *P. putida* (Molina-Santiago et al. 2014; Kim et al. 2017). However, there is limited knowledge on the effect of indole on drug resistance in *Vibrio* sp., which is a main bacterial pathogen of aquatic organisms. In our previous work, indole was detected in the supernatant of *V. splendidus* grown to stationary phase at a concentration of 125 μM (Zhang et al. 2017). This level of indole is similar to that in *E. coli* cultures at stationary phase (Li and Young 2013; Gaimster et al. 2014). In this study, 125 μM exogenous indole increased tetracycline resistance

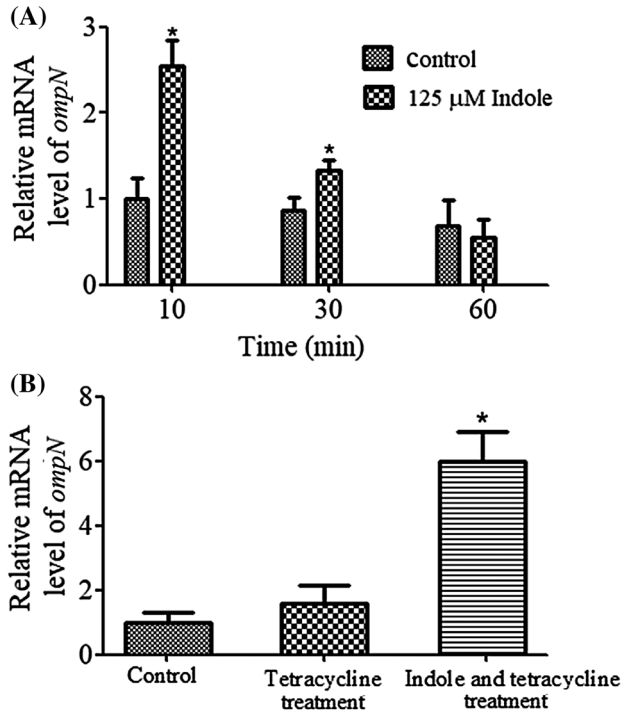

Fig. 6 mRNA level of the *ompN* gene determined using real-time RT-PCR. **a** The expression of the *ompN* gene in the presence of indole at different time points. *V. splendidus* was cultured at 28 °C to an OD_{600} of 0.2 and was continuously cultured without indole or with 125 μM indole for another 10 min, 30 min and 60 min. **b** mRNA level of *ompN* gene in the presence of tetracycline or a mixture of tetracycline and indole. *V. splendidus* grew at 28 °C to an OD_{600} of 0.2. Cells were incubated in medium supplemented with 20 μg/mL tetracycline or a mixture of 20 μg/mL tetracycline and 125 μM indole. *V. splendidus* cultured with ethanol was used as a control. Total RNA was extracted and used for real-time RT-PCR. The mRNA level of the *ompN* gene was normalized to that of the 16S rDNA gene. The data are the means for at least three independent experiments and are presented as the means ± SD

by over 4.5-fold in *V. splendidus*, while 0.25–4.0 mM indole induced AMR in *S. enterica*, *A. tumefaciens* and *Salmonella typhimurium* (Nikaido et al. 2012; Vega et al. 2012; Molina-Santiago et al. 2014; Lee et al. 2015). To our knowledge, this is the first study to investigate the effect of indole on antibiotic resistance in *V. splendidus*.

Mechanisms of bacteria acquiring antibiotic resistance include reduction in the permeability of the cell membrane, activation of efflux of the antimicrobial agents, mutation in the target site, modification or degradation of the antimicrobial agents and acquisition of alternative metabolic pathways to avoid the antimicrobial agents (Mcdermott et al. 2003). Tetracycline can pass through the OM by two pathways: a lipid-mediated pathway for hydrophobic antibiotics, and general diffusion porins for hydrophilic antibiotics (Delcour 2009). OMPs have been reported to be involved in the tetracycline and ampicillin resistance in *E. coli* and the decreased expression of the *ompF* was responsible for the tetracycline resistance in *E. coli* and many other bacteria (Xu et al. 2006; Delcour 2009). In our present study, the differentially expressed protein that was upregulated was identified as OmpN. This outer membrane protein has been cloned in *Vibrio cyclitrophicus*, *Vibrio crassostreae*, *V. vulnificus*, and *Vibrio alginolyticus*, etc., but its defined function has never been explored. The upregulation of the OmpN in the presence of tetracycline suggested that OmpN may function as a component of an efflux system to pump tetracycline out of the cells, thus leading to the increase tetracycline resistance in *V. splendidus*. Indole is known to affect bacterial antibiotic resistance in different ways. Indole increased antibiotic resistance by modulating efflux pumps in *P. aeruginosa*, *P. putida* and *A. tumefaciens* (Molina-Santiago et al. 2014; Lee et al. 2015). Similarly, the increased tetracycline resistance and the upregulation of the *ompN* gene by indole and the mixture of indole and tetracycline indicated that indole increased tetracycline resistance of *V. splendidus* via upregulation of the porin OmpN, a probable component of an efflux system in *V. splendidus*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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