

ORIGINAL ARTICLE

Comparative transcriptome and phenotype analysis revealed the role and mechanism of *ompR* in the virulence of fish pathogenic *Aeromonas hydrophila*

Mengmeng Zhang¹ | Jianping Kang² | Bin Wu² | Yingxue Qin^{1,3}  | Lixing Huang¹ | Lingmin Zhao¹ | Leilei Mao¹ | Suyun Wang¹ | Qingpi Yan¹ 

¹Fisheries College, Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, Jimei University, Xiamen, China

²Fujian Fisheries Technology Extension Center, Fuzhou, China

³Fujian Province Key Laboratory of Special Aquatic Formula Feed, Fujian Tianma Science and Technology Group Co., Ltd., Fuqing, China

Correspondence

Yingxue Qin and Qingpi Yan, Fisheries College, Jimei University, Xiamen, China. Emails: yxqin@jmu.edu.cn (YQ); Yanqp@jmu.edu.cn (QY)

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Abstract

Aeromonas hydrophila B11 strain was isolated from diseased *Anguilla japonica*, which had caused severe gill ulcers in farmed eel, causing huge economic losses. EnvZ-OmpR is a model two-component system in the bacteria and is widely used in the research of signal transduction and gene transcription regulation. In this study, the *ompR* of *A. hydrophila* B11 strain was first silenced by RNAi technology. The role of *ompR* in the pathogenicity of *A. hydrophila* B11 was investigated by analyzing both the bacterial comparative transcriptome and phenotype. The qRT-PCR results showed that the expression of *ompR* in the *ompR*-RNAi strain decreased by 97% compared with the wild-type strain. The virulence test showed that after inhibition of the *ompR* expression, the LD₅₀ of *A. hydrophila* B11 decreased by an order of magnitude, suggesting that *ompR* is involved in the regulation of bacterial virulence. Comparative transcriptome analysis showed that the expression of *ompR* can directly regulate the expression of several important virulence-related genes, such as the bacterial type II secretion system; moreover, *ompR* expression also regulates the expression of multiple genes related to bacterial chemotaxis, motility, adhesion, and biofilm formation. Further studies on the phenotype of *A. hydrophila* B11 and *ompR*-RNAi also confirmed that the downregulation of *ompR* expression can decrease bacterial chemotaxis, adhesion, and biofilm formation.

KEYWORDS

Aeromonas hydrophila, comparative transcriptome, *ompR*, RNAi, virulence

1 | INTRODUCTION

Aeromonas hydrophila is widely distributed throughout various foods, soils, sea water, and fresh water (Ghatak et al., 2016; Navarro-Garcia, Serapio-Palacios, Vidal, Salazar, & Tapia-Pastrana, 2014; Qin et al., 2014). It can cause disease outbreaks in aquatic

animals and lead to high mortality (Qin, Lin, Chen, Xu, & Yan, 2016; da Silva et al., 2012). Moreover, in 1970, this bacterium was reported to cause human infectious diarrhea (Helm & Stille, 1970), and it has been described as one of the most common pathogens that cause severe public health problems (Igbiosa, Igumbor, Aghdasi, Tom, & Okoh, 2012). The pathogenicity and pathogenic

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mechanism of *A. hydrophila* have also been reported (González-Serrano, Santos, García-López, & Otero, 2002; Li, 2011). Current research generally assumes that the pathogenicity of *A. hydrophila* is closely related to the complexity of its virulence factors, and moreover, different virulence factors cooperate with each other to jointly exert their pathogenic role (Shemesh, Tam, & Steinberg, 2007; Yu et al., 2005). However, the pathogenic mechanism of *A. hydrophila* has not been fully described to date, and the literature is even contradictory (Chandrarathna et al., 2018; Van der Marel, Schroers, Neuhaus, & Steinhagen, 2008). Our team have been tracking the bacterial diseases of farmed eel in Fujian China for about 20 years and isolated dozens of strains of *A. hydrophila*. Among them, the B11 strain is particularly virulent, causing severe gill ulcers in farmed eel in Fujian and bringing huge economic losses.

The two-component system EnvZ/OmpR mainly regulates bacterial osmotic pressure. EnvZ is a transmembrane histidine kinase located in the inner membrane of bacteria. It can monitor changes in osmotic pressure in the environment. OmpR is a response regulator, which is essentially a transcription factor located in the promoter region of membrane porin. OmpR activated by EnvZ and binds to specific DNA regions to activate the expression of corresponding genes such as *ompF* and *ompC* (Gagan, Tanuja, & Aparna, 2012). Thereby, it regulates a variety of cellular functions including motility, biofilm formation, adaptation to acidic conditions, and toxicity (Bang, Audia, Park, & Foster, 2002; Bontemps-Gallo et al., 2019; Jubelin et al., 2005; Mattison, Oropeza, & Kenney, 2002; Silva et al., 2018). It has been shown that OmpR regulates the flagellar pathway, the ability of bacteria to move, and the secretion of antibiotics in the bacteria *Xenorhabdus nematophila* (Park & Forst, 2006). In *Escherichia coli*, *ompR* inactivation affects the expression of more than 100 genes, thus causing changes in many bacterial physiological functions such as motility, biofilm formation, adaptability to acidic environments, and even pathogenicity (Kakuda et al., 2014). The latest researches revealed the two-component system EnvZ/OmpR could affect the pH and the virulence of *Vibrio cholerae* and also affect the tolerance of bacteria to ethanol (Xi et al., 2019; Zhang, Ye, et al., 2018).

Although the researches on EnvZ/OmpR have been extensive, there are few reports on the function of this two-component system from the transcriptome level. And the function of EnvZ/OmpR in aquatic animal pathogen *Aeromonas* remains unknown. Comparative transcriptomics can reveal the function of genes more fully from mRNA level, which help to deeply understand the function of genes.

Since OmpR is the transcriptional regulatory factor of the two-component system EnvZ/OmpR and plays an important role in the pathogenicity of many bacteria, it is important to investigate the role it plays in the pathogenesis of *A. hydrophila* (Reboul et al., 2014), and in this study, the expression of *ompR* was silenced in pathogenic *A. hydrophila* B11 by RNAi technology. The phenotypes and transcriptome of wild-type and silent strains were compared to explore the role of *ompR* in the *A. hydrophila* pathogenesis.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and growth conditions

Escherichia coli growth conditions: 37°C, 220 rpm; *A. hydrophila* B11 growth conditions: 28°C, 220 rpm. The antibiotic chloramphenicol was added to the LB medium (pH = 7.0) at a concentration of 34 µg/ml. The strains and plasmids used in this study are listed in Table A1.

2.2 | Stable gene silencing

The expression of *ompR* was stably silenced following previously described methods (Qin et al., 2014; Tokunaga et al., 2015; Zhang, Luo, et al., 2018; Zhang, Ye, et al., 2018; Zhang et al., 2019; Zhang, Yan, et al., 2018). The designed synthetic shRNA was annealed to form a double strand, which was then ligated to the pACYC184 plasmid after digestion with the enzymes *SphI* and *BamHI* to form pACYC184-*ompR*. The recombinant plasmid was introduced into *A. hydrophila* by electroporation, and the stable-silencing strain *ompR*-RNAi was screened by chloramphenicol and qRT-PCR. The shRNAs are listed in Table A2.

2.3 | RNA extraction and reverse transcription

The total bacterial RNA was extracted using the TRIzol method (Invitrogen) according to the manufacturer's recommended protocol; the first-strand cDNA was synthesized from the total RNA and was synthesized using the PrimeScript™ RT reagent kit with the gDNA Eraser kit (Takara), following the manufacturer's recommendations.

2.4 | qRT-PCR

qRT-PCR was performed on a QuantStudio™ 6 Flex real-time PCR system (ABI) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Threshold cycles and dissociation curves were determined with QuantStudio™ 6 Flex software to confirm that only one PCR product was amplified and detected. Gene expression levels were normalized to 16S rRNA. The Relative Expression Software Tool (version 2, REST 2008) was used to calculate the relative expressions of genes in qRT-PCR using the Pair Wise Fixed Reallocation Randomization Test (Pfaffl, Horgan, & Dempfle, 2002). The utilized mathematical model was based on the mean crossing point deviation between the sample and the control group, which was normalized by the mean crossing point deviation of the reference genes. Specific amplification efficiencies were included in the correction of the quantification ratio. Differences between groups were assessed by ANOVA followed by Tukey's LSD (Wang et al., 2019; Zhang, Luo, et al., 2018; Zhang, Ye, et al., 2018; Zhang, Yan, et al., 2018). The primers are listed in Table A3.

2.5 | Transcriptome sequencing and data analysis

Samples of wild-type strain B11 and *ompR*-RNAi strain ($n = 3$) were sent to Shanghai Majorbio company, where Illumina Hiseq was used for transcriptome sequencing (Levin et al., 2010; Parkhomchuk et al., 2009). To ensure the accuracy of the subsequent biological information analysis, the original sequence data were analyzed and filtered by Majorbio Pharmaceutical Technology Co., Ltd. These high-quality sequences were compared with the specified reference genome by using Bowtie software (Version 2.2.9), and different genes were selected. The selecting conditions were $p \leq .05$ and $|\log_{2}FC| \geq 1$, that is $p \leq .05$, or the difference multiple exceeding 2 (Trapnell et al., 2013). In this study, the gene selection is based on the principles of phenotype-related and significant differences in RNA-seq analysis. The Gene Ontology (GO) database was used to classify the functions of different genes, and software Go tools were used for functional enrichment analysis (Lu, Peatman, Tang, Lewis, & Liu, 2012; Tang et al., 2008). The software KOBAS was used for KEGG PATHWAY enrichment analysis, Fisher's exact test was used for calculation, and the BH (FDR) method was used for multiple tests (Trapnell et al., 2010; Xie et al., 2011). The relationship between genes was established using GeneMANIA with reference to *E. coli*, and the relationship map was constructed using Cytoscape.

2.6 | Virulence test

Healthy zebrafish of equal weight (0.5 ± 0.02 g) and size (3.5 ± 0.2 cm) were obtained from Yudu Aquarium Xiamen and were kept for one week under pathogen-free laboratory conditions at a water temperature of 28°C. Bacteria were harvested in the early exponential phase, and suspensions were adjusted to 10^8 , 10^7 , 10^6 , 10^5 , and 10^4 cfu/ml. Then, 50 μ l bacteria suspension was injected into the base of the pectoral fin of zebrafish. Fish of the control group were injected with phosphate buffer solution (PBS). Each gradient was conducted three times in parallel, and 30 fish were used for each gradient. The numbers of diseased zebrafish were recorded in the following 96 hr, and the LD₅₀ of B11 strain and *ompR*-RNAi strain were calculated by the method of Reed-Muench (Reed & Muench, 1938).

2.7 | Chemotaxis

The concentration of the overnight bacterial suspension was adjusted to OD₆₀₀ = 1.0 using PBS. A capillary tube with an inner diameter of 0.1 mm was sealed at one end, filled with mucus, and dipped into a bacterial suspension. After incubation for 1 hr, the liquid in the capillary was blown out for a dilution coating count to determine the number of bacteria in the capillary. Three trials were conducted for each group.

2.8 | Motility

The concentration of the overnight bacterial suspension was adjusted to OD₆₀₀ = 0.2 with PBS. One micro liter of the bacterial suspension was spotted onto the center of LB plates (0.3% agar), and the plates were incubated at 28°C for 12 hr, after which the diameters of the colonies were measured and recorded (Zuo et al., 2019).

2.9 | Adhesion assay

Bacterial adhesion assays were performed using the method described by Liu et al. (2017) and Kong et al. (2015). A volume of 20 μ l of mucus was evenly spread onto a glass slide and fixed with methanol for 30 min. Then, 1 ml of bacterial suspension (10⁸ cfu/ml) was placed on the mucus-coated glass slides, incubated for 2 hr at 28°C in a humidified chamber, and then, the surface bacteria were washed three times with PBS. Finally, the bacteria were

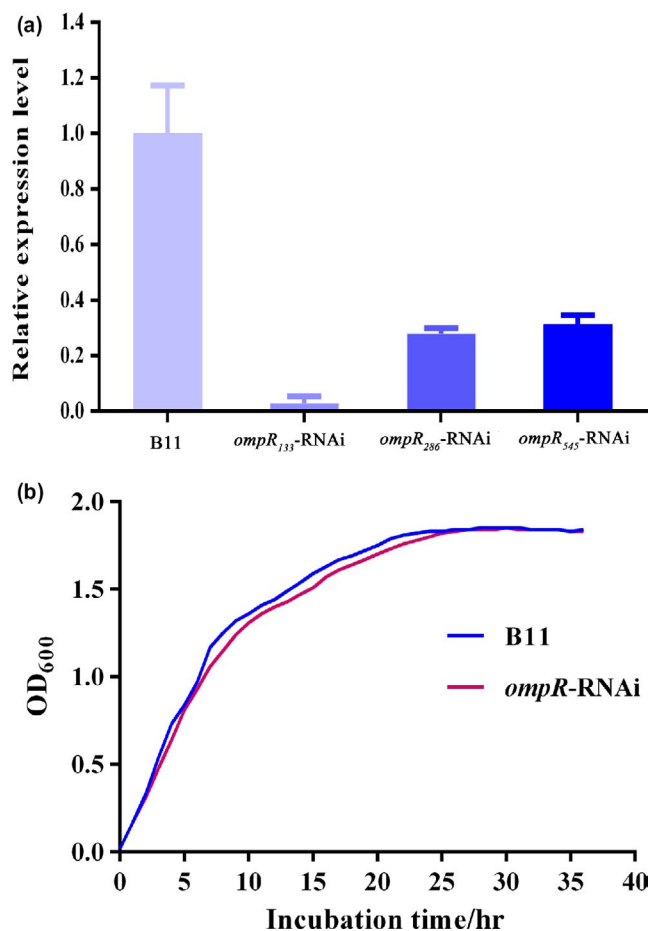


FIGURE 1 Construction of silenced strain of *A. hydrophila* B11. (a) Expressions of *ompR* in B11 strain and *ompR*-RNAi strain. (b) Growth curves of B11 strain and *ompR*-RNAi strain. Data are presented as means \pm SD of three independent biological replicates. ** $p < .01$

fixed with 4% methanol for 30 min, dyed with crystal violet for 3 min, and counted under a microscope (Leica) ($\times 1,000$). Five trials were conducted per group, and 20 fields of view were selected.

2.10 | Biofilm formation

Bacterial biofilms were formed following the method described by Huang et al. (2018) and Luo et al. (2016). The concentration of the overnight bacterial suspension was adjusted to $OD_{600} = 0.2$. 100 μ l of bacterial suspension was added to a 96-well plate and incubated at 28°C for 24 hr. Then, the 96-well plate was washed with sterile PBS three times and stained with 200 μ l 1% crystal violet for 15 min. After that, the 96-well plate was rinsed again with sterile PBS and then air-dried. Finally, 200 μ l acetic acid (33%) was added to solubilize the stained biofilm, which was quantitated by measuring OD_{590} nm. Six replicates were performed for each treatment.

3 | RESULT

3.1 | Construction of the *ompR*-RNAi strain

Figure 1a shows that the expressions of *ompR* in *ompR*₁₃₃-RNAi, *ompR*₂₈₆-RNAi, and *ompR*₅₄₅-RNAi decreased by 97%, 72%, and 68% compared with that of B11. Therefore, the *ompR*₁₃₃-RNAi (named *ompR*-RNAi) was selected for further studies. The growth curves in Figure 1b show that the *ompR*-RNAi strain displayed almost the same growth rate as the B11 strain, indicating that the expression of *ompR* does not affect the growth of *A. hydrophila*.

This indicated that the results of subsequent biological tests were not caused by differences in the growth between both strains.

3.2 | Effect of *ompR* on the virulence of *A. hydrophila*

The network of important virulence-related genes of *A. hydrophila* B11 (Figure 2a) showed that *ompR* can regulate the expression of multiple virulence genes, such as *gspH*, *gspG* and *ffh*, to regulate the bacterial virulence. The results in Figure 2b suggested that *ompR* positively regulates *gspG* and *gspH* and negatively regulates *hns*, *hlyA*, *ast*, and *ffh*. Furthermore, the results of the virulence test showed that zebrafish that were injected with *ompR*-RNAi or with B11 died within 48 hr (Figure 2c). According to the data in Figure 2d and the method of ReeD-Muench, the LD_{50} for *ompR*-RNAi strain and the wild-type strain B11 were 2.21×10^5 cfu/ml and 2.98×10^4 cfu/ml, respectively. This indicated that after the expression of *ompR* was inhibited, the LD_{50} of *A. hydrophila* B11 decreased by an order of magnitude, which suggested that *ompR* was likely involved in the regulation of bacterial virulence.

3.3 | Bacterial chemotaxis and motility

The network (Figure 3a) showed that *ompR* can regulate the expression of multiple chemotaxis and motility-related genes, thus regulating the chemotaxis of *A. hydrophila*. The results shown in Figure 3b suggested that *ompR* positively regulated *motA* and *cheV* and negatively regulated *cheY* and *fliD*. The phenotype results showed that after the expression of *ompR* was inhibited, the

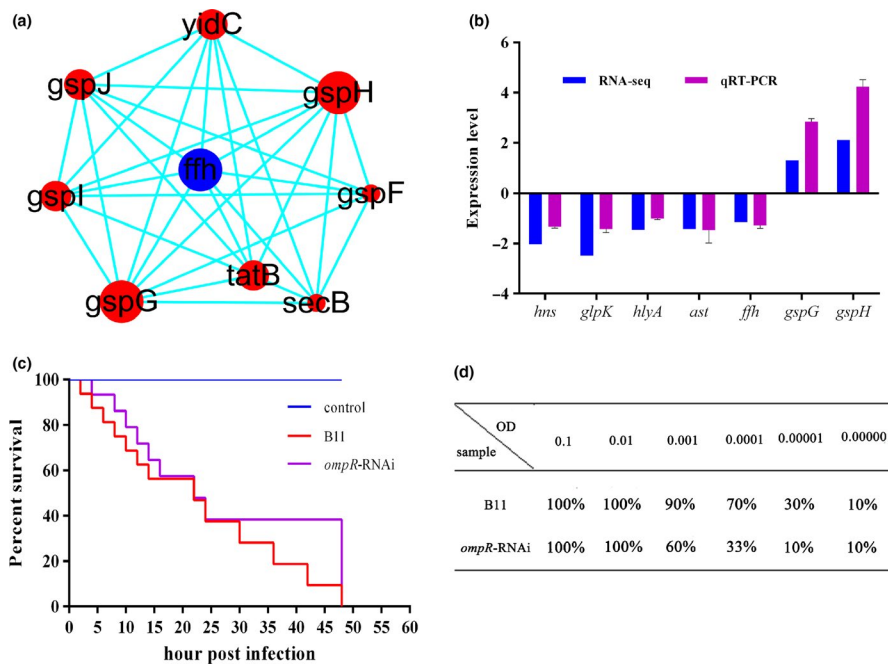


FIGURE 2 Effect of *ompR* expression on the virulence of *A. hydrophila*. (a) Network of important virulence genes regulated by *ompR* (red indicates significantly upregulated, and blue indicates significantly downregulated genes; the circle size indicates the amount of gene expression). (b) Expression of important virulence genes in *ompR*-RNAi. (c) Survival rate of fish after bacterial injection. (d) Mortality rate of fish after bacterial injection. Data are presented as means \pm SD from three independent biological replicates. ** $p < .01$

ability for bacterial chemotaxis decreased by 45.5% (Figure 3c); however, the bacterial motility did not change (Figure 3d,e).

ompR was inhibited, the biofilm formation ability of *ompR*-RNAi decreased by 15.1% (Figure 5c,d).

3.4 | Bacterial adhesion

The network of adhesion in Figure 4a showed that *ompR* can regulate the expressions of multiple adhesion genes. These genes are mainly involved in the energy metabolism of bacteria, which affects bacterial adhesion. The results shown in Figure 4a,b suggested that *ompR* negatively regulates most of the adhesion-related genes. The phenotype results in Figure 4c,d showed that the adhesion ability of *ompR*-RNAi strain decreased by 14.9%.

3.5 | Bacterial biofilm formation ability

The results in Figure 5 showed that *ompR* can regulate the expression of multiple biofilm formation-related genes (Figure 5a). These genes play a major role in aerobic or anaerobic metabolism and bacterial energy regulation. The result in Figure 5b suggested that *ompR* positively regulated the expressions of *glpA*, *glpB*, *glpD*, and *trpE*. The phenotype results showed that after the expression of

4 | DISCUSSION

The results of this study showed that the virulence of *A. hydrophila* B11 decreased after the expression of the *ompR* gene was inhibited. Comparative transcriptome analysis suggested that multiple virulent genes, such as *hlyA*, *hns*, *ast*, *ffh*, *gspH*, and *gspG*, were regulated by *ompR*. Ffh is a major protein component of signal recognition particles (SRP) and plays an important role in mediating the co-expression of both the cell membrane and secreted proteins (Mishra et al., 2019; Venkatesan, Palaniyandi, Sharma, Bisht, & Narayanan, 2018). GspG and GspH belong to the type II secretion system, the main function of which is to participate in the secretion of virulence proteins, which is itself affected by the Sec translocon pathway, regulated by *ffh* (Cianciotto & White, 2017). The type II secretion system plays a pivotal role in the pathogenesis of bacteria (Genin & Boucher, 2002, 2004; Howard et al., 2019). Therefore, this suggests that OmpR can regulate the virulence of *A. hydrophila* B11 by regulating the expression and function of the bacterial type II secretion system.

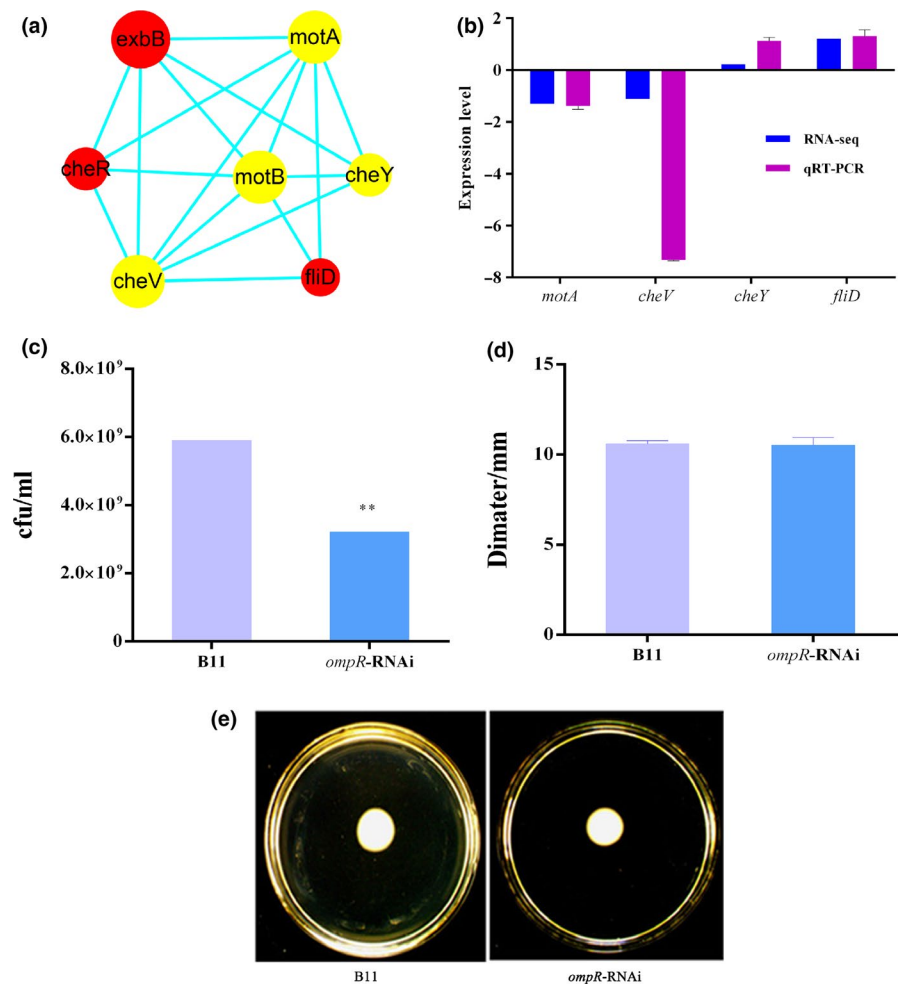


FIGURE 3 Effect of *ompR* expression on chemotaxis and motility of *A. hydrophila*. (a) Network of chemotaxis and motility-related genes regulated by *ompR* (red is significantly upregulated, blue is significantly downregulated, and yellow is not significant; the circle size indicates the amount of gene expression). (b) Expressions of several chemotaxis and motility-related genes in *ompR*-RNAi. (c) Chemotaxis capacity of B11 strain and *ompR*-RNAi strain. (d) Colony diameter of B11 and *ompR*-RNAi strain. (e) Colony of B11 and *ompR*-RNAi strain on plates. Data are presented as means \pm SD from three independent biological replicates. ** indicates $p < .01$

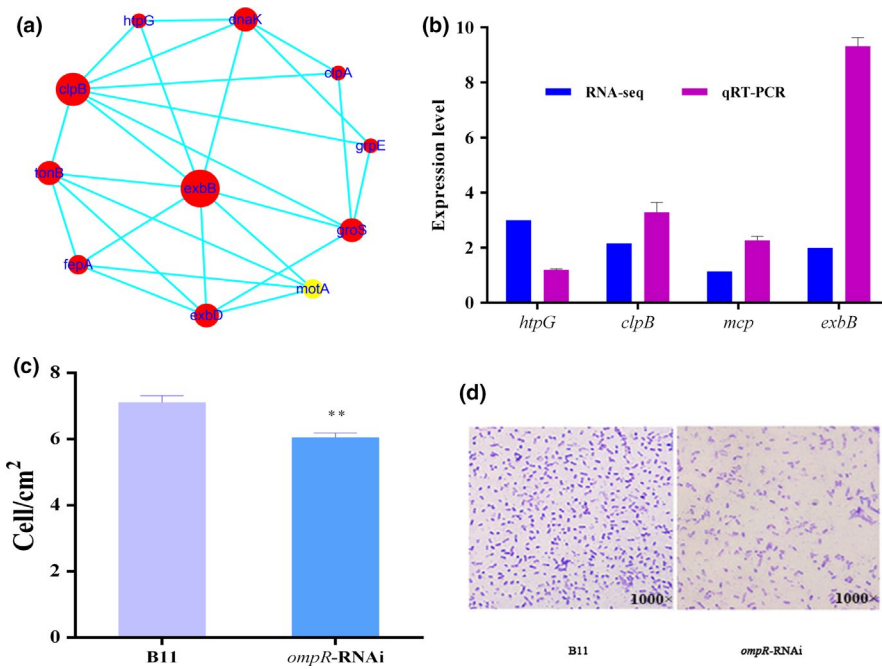


FIGURE 4 Effect of *ompR* expression on adhesion of *A. hydrophila*. (a) Network of adhesion genes regulated by *ompR* (red is significantly upregulated, and yellow is significantly downregulated; the circle size indicates the amount of gene expression). (b) Expressions of several adhesion-related genes in *ompR*-RNAi. (c) Adhesion ability of B11 strain and *ompR*-RNAi strain. (d) Adhesive bacteria under the microscope. Data are presented as means \pm SD of three independent biological replicates. ** $p < .01$

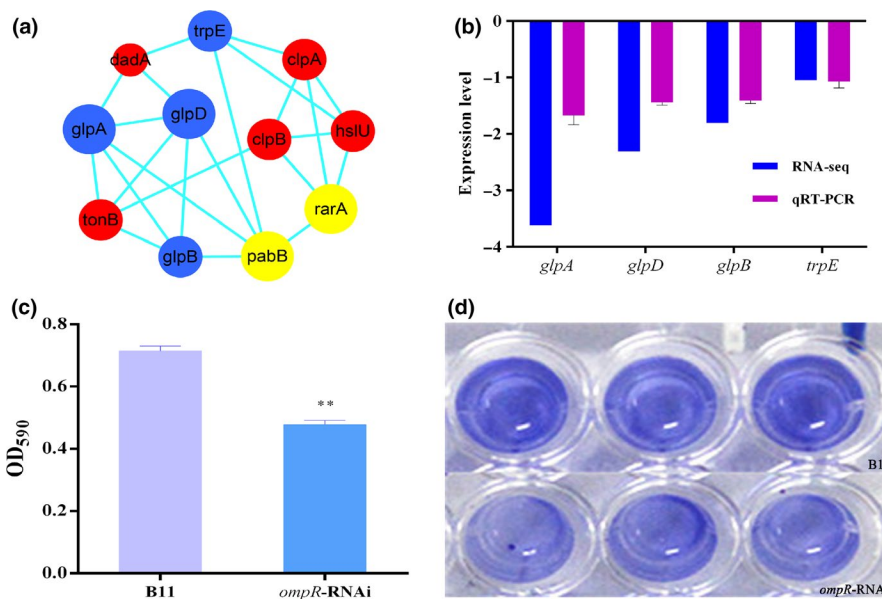


FIGURE 5 Effect of *ompR* expression on *A. hydrophila* biofilms. (a) Network of biofilm formation-related genes that are regulated by *ompR* (red is significantly upregulated, blue is significantly downregulated, and yellow is not significant; the circle size indicates the amount of gene expression). (b) Expressions of several biofilm formation-related genes in *ompR*-RNAi. (c) Biofilm formation ability of B11 and *ompR*-RNAi. (d) Bacteria biofilm formation in a 96-well plate. Data are presented as means \pm SD of three independent biological replicates. ** $p < .01$

To further identify the mechanism of OmpR in the regulation of the *A. hydrophila* virulence, several biological characteristics of wild-type strain and *ompR*-RNAi strain were compared. The results showed that the chemotaxis, adhesion, and biofilm ability of bacteria decreased significantly (Kunkle, Bina, Bina, & Bina, 2019; Tipton & Rather, 2017). This indicated that OmpR can regulate various physiological functions.

Studies have shown that chemotaxis (as a navigational system of motion) has been identified as an important feature of the virulence of many pathogens (Freter, Allweiss, O'Brien, Halstead, & Macsai, 1981). In *E. coli*, inactivation of the chemotactic signaling protein CheW leads to a decrease in the directionality of bacterial movement (Macnab, 1996; Stock, 1996). A study on *Ralstonia solanacearum* showed that *cheW* deletion decreases both pathogenicity and parasitism (Yao & Allen, 2006). A study of *Vibrio*

alginolyticus (ND-01) showed that mutations of *mcp*, *aer*, *cheV*, and *cheB* affect the expression of downstream genes, the chemotaxis of bacteria, and the virulence of bacteria (Huang et al., 2017). The CheY function is related to signal transduction, and when environmental signals change, this signal is transmitted to the flagellar movement by CheY (Stock, Robinson, & Goudreau, 2000; West & Stock, 2001). A study of *E. coli* showed that chemotaxis signaling affects the expression of *motA/B* (Morimoto, Che, Minamino, & Namba, 2010). MotA/B and FlhD consist of bacterial flagellin and affect the movement of bacteria; MotB is the major regulatory protein in MotA/B (Pourjaberi, Terahara, Namba, & Minamino, 2017; Tang et al., 2010). In this study, when the expression of *ompR* was inhibited, chemotaxis of *A. hydrophila* B11 decreased; however, no change of bacterial motility was detected. This indicates that

OmpR regulates bacterial chemotaxis by regulating the capability of bacteria to respond to environment signals, rather than their motility.

When bacteria adhered to the surface of objects, they can secrete extracellular polymers to enhance their adhesion ability, which leads to infection. Therefore, adhesion is typically regarded as an important virulence factor of bacteria. A study in *Pantoea alhagi* showed that OmpR and LrhA can affect bacterial colonization (Li, Liang, et al., 2019). Skorek et al. (2013) reported that OmpR positively regulated the expression of FlhDC in *Yersinia enterocolitiss* O: 9, which FlhDC further affected bacterial adhesion and biofilm formation. Transcriptome analysis further suggested that *clpB* and *exbB* may play key roles in the adhesion of *A. hydrophila* B11. *clpB* is a member of the AAA + ATP superfamily and participates in the energy metabolism (Suarez et al., 2008). *exbB*, a member of the TonB system, can transport nutrients and affect the virulence of bacteria (Abdollahi, Rasooli, & Gargari, 2018). This suggests that OmpR regulates bacterial adhesion by regulating nutrient transport and energy metabolism, which further affects the virulence of *A. hydrophila*.

It is well known that biofilms can help bacteria to colonize, change their ability to adhere, and cause disease (Li, Peppelenbosch, & Smits, 2019). OmpR was found to regulate the expression of LrhA in *Pantoea alhagi* and regulate bacterial both biofilm formation and colonization (Li, Liang, et al., 2019). Studies of other bacteria also found that biofilm formation decreased when the mRNA expression of *ompR* was inhibited (Lin et al., 2018; Shi et al., 2018). The transcriptome analysis of this study suggested that *ompR* can positively regulate the expression of multiple genes related to biofilm formation, such as *glpA*, *glpB*, and *glpD*. In *E. coli*, *glpA* and *glpB* encode anaerobic enzymes, while *glpD* encodes aerobic enzymes, both of which are important enzymes in the bacterial metabolism (Ehrmann, Boos, Ormseth, Schweizer, & Larson, 1987). It has been speculated that *ompR* regulates bacterial biofilm formation by regulating the energy metabolism of bacteria. The results of the present study confirmed the results in *A. vickers* (Zhang, 2017).

5 | CONCLUSION

In conclusion, the results suggested that (a) *ompR* can regulate the virulence of *A. hydrophila* B11 by directly regulating the expression and function of the bacterial type II secretion system; (b) *ompR* regulates bacterial chemotaxis by regulating the capability of bacteria to respond to environment signals, rather than their motility; (c) *ompR* regulates bacterial adhesion by regulating nutrient transport and energy metabolism; and (d) *ompR* regulates bacterial biofilm formation by regulating the energy metabolism.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Mengmeng Zhang: Data curation (equal); Formal analysis (equal); Methodology (equal); Software (lead); Writing-original draft (lead); Writing-review & editing (equal). **Jianping Kang:** Funding acquisition (supporting); Project administration (lead); Resources (equal); Supervision (equal). **Bin Wu:** Funding acquisition (supporting); Project administration (equal); Resources (equal); Supervision (equal). **Yingxue Qin:** Data curation (lead); Formal analysis (equal); Funding acquisition (lead); Methodology (equal); Project administration (lead); Resources (equal); Supervision (lead); Writing-original draft (equal); Writing-review & editing (equal). **Lixing Huang:** Methodology (supporting); Software (supporting); Supervision (supporting). **Lingmin Zhao:** Formal analysis (supporting); Investigation (supporting). **Leilei Mao:** Methodology (supporting); Validation (supporting). **Suyun Wang:** Investigation (supporting); Validation (supporting). **Qingpi Yan:** Conceptualization (equal); Funding acquisition (supporting); Methodology (supporting); Project administration (equal); Resources (supporting); Supervision (equal).

ETHICS STATEMENT

All laboratory animals were operated on according to the guidelines in the "Guidelines for the Care and Use of Laboratory Animals" developed by the National Institutes of Health. The animal experiments were approved by Jimei University Animal Ethics Committee (Acceptance NO: JMULAC201159).

DATA AVAILABILITY STATEMENT

The transcriptome data have been deposited to SRA database under the BioProject accession number PRJNA595449: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA595449>

ORCID

Yingxue Qin  <https://orcid.org/0000-0001-7850-7010>

Qingpi Yan  <https://orcid.org/0000-0001-8698-3121>

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APPENDIX 1

Strain or plasmid	Genotype and/or phenotype	Source or reference
Plasmid		
pACYC184	(Cm ^R Tc ^R)	Provided by Prof. Niepin
Strains		
B11	Wild-type strain (Sm ^R), isolated from diseased <i>Anguilla japonica</i>	Isolated and keep in our lab
pACYC184- <i>ompR</i>	pACYC184 derivative containing 60 bp fragment of one short-hairpin RNA sequence targeting the coding region of <i>ompR</i> mRNA and ORF (Cm ^R)	This study
<i>ompR</i> -RNAi	<i>ompR</i> was silenced by shRNA (Sm ^R , Cm ^R)	This study
<i>E. coli</i> DH5α	F ⁻ , φ80 <i>lacZ</i> ΔM15, Δ(<i>lacZYA-argF</i>) U169, <i>deoR</i> , <i>recA1 endA1</i> , <i>hsdR17</i> (rK ⁻ , mK ⁺), <i>phoA</i> , <i>supE44</i> , λ ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Takara, Dalian, China

Abbreviations: CmR, chloramphenicol-resistant; SmR, strepto- mycin-resistant; TcR, tetracycline-resistant.

TABLE A1 Strains and plasmids used in this study

Primer	shRNA sequence
<i>ompR</i> ₁₃₃ -F	5'-GATCCACCCGAGAGAAGTTCAGCCTGTTCAAGAGACAGGC TGAAGTTCTCTCGGGT TTTTTGCATG-3'
<i>ompR</i> ₁₃₃ -R	5'-CAAAAAAACCAGAGAGAAGTTCAGCCTGTCTCTTGAACAG GCTGAAGTTCTCTCGGGT-3'
<i>ompR</i> ₂₈₂ -F	5'-GATCCGCCCTACACCTATCTGGAAGATTCAAGAGATCTTCC AGATAGGTGTAGGGCTTTTTGCATG-3'
<i>ompR</i> ₂₈₂ -R	5'-CAAAAAAGCCCTACACCTATCTGGAAGATCTTGAATCTT CCAGATAGGTGTAGGGCG-3'
<i>ompR</i> ₅₄₅ -F	5'-GATCCGCGACAAACTGATGAACCTGGTTCAAGAGACCAGGT TCATCAGTTTGTCTGCTTTTTGCATG-3'
<i>ompR</i> ₅₄₅ -R	5'-CAAAAAAGCGACAAACTGATGAACCTGGTTCAAGAGACCA GTTTCATCAGTTTGTCTGCG-3'

Note: Sequences specifying restriction endonuclease cleavage sites are underlined.

TABLE A2 Oligonucleotides for the stabilization of gene-silencing shRNA

TABLE A3 Primers for qRT-PCR

Primer	Nucleic acid sequence	Primer	Nucleic acid sequence
<i>ompR</i> -R	GATGGTGATCCGGTGGCA	<i>motA</i> -R	GCTGCTGGACGTGGAGTTG
<i>ompR</i> -F	ACGAATACATAGCCCAGACCC	<i>motA</i> -F	GCATCCTTGCCGTTGGTG
<i>16s</i> -R	TTCTGATTCCCGAAGGCAC	<i>cheY</i> -R	CAACACCCACGAAGCAG
<i>16s</i> -F	GGAGCAAACAGGATTAGATACCC	<i>cheY</i> -F	TCGGCAGTCACCATCAG
Primer	Nucleic acid sequence	Primer	Nucleic acid sequence
<i>hns</i> -R	AAGGCGACCAAGAGCAAA	<i>fliD</i> -R	AGGTGGTTGCGGTGTTTG
<i>hns</i> -F	AAAGTCTTCGACGGATTACC	<i>fliD</i> -F	CGACTCCTTGCGGCTTAC
<i>hlyA</i> -R	TGGGAGTGAAAAGCGGACAG	<i>gspG</i> -R	GCGTCGTC AATCGGGTTT
<i>hlyA</i> -F	CGGGTGTAGGAGGTCAGGGT	<i>gspG</i> -F	GGTCGGGTAGCGGTTGTT
<i>ast</i> -R	TGCCGGAGAACGACCACT	<i>gspH</i> -R	GCCGTCACCCTCAGCAT
<i>ast</i> -F	CCTGGCTCGCTTCTAGGGT	<i>gspH</i> -F	CTGCCAGCCGTTGTCCT
<i>ffh</i> -R	AAGGTGCTGGTAGTCAGTGCG	<i>glpB</i> -R	CCTTATCAGCAGGACTACTCGC
<i>ffh</i> -F	TGGTGGCGTGAAGGTGTTT	<i>glpB</i> -F	GGACTCGTCATCGCACAGC
<i>htpG</i> -R	AGCGAGTTTGCAATGTGC	<i>trpE</i> -R	TGCAACGGGAGCTTGATTG
<i>htpG</i> -F	GTTCTTGCGGCAGCGTA	<i>trpE</i> -F	GCGATGTGGTCGATGGTGAT
<i>clpB</i> -R	CACCACTCCGAGAAAGATTACG	<i>cheV</i> -R	GCTGCTGGACGTGGAGTTG
<i>clpB</i> -F	GCTTCGCTCACCTCCACCTC	<i>cheV</i> -F	GCATCCTTGCCGTTGGTG
<i>exbB</i> -R	GGCATGGTGCAGGTGTTT	<i>glpD</i> -R	ACAACCGCATCGTCTTCGTC
<i>exbB</i> -F	TCAGGTGTCTTCCAGCTTCT	<i>glpD</i> -F	GGACTCGTCATCGCACAGC
<i>glpA</i> -R	TCGAGGTAGAGTGCCAGGAGG		
<i>glpA</i> -F	TGGTGCCGATGAGGGAGA		