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## SPECIALTY SECTION

This article was submitted to  
Marine Fisheries, Aquaculture and  
Living Resources,  
a section of the journal  
Frontiers in Marine Science

RECEIVED 13 September 2022

ACCEPTED 06 December 2022

PUBLISHED 19 December 2022

## CITATION

Su W, Deng Y, Zang S, Gao S and  
Feng J (2022) The global role of Lrp in  
*Vibrio alginolyticus* and its response to  
diverse physicochemical factors.  
*Front. Mar. Sci.* 9:1042899.  
doi: 10.3389/fmars.2022.1042899

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# The global role of Lrp in *Vibrio alginolyticus* and its response to diverse physicochemical factors

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Leucine-responsive regulatory protein (Lrp) is an essential transcriptional regulator in prokaryotes. However, the regulatory role of *lrp* in *Vibrio alginolyticus* has still not been studied in detail. In this study, an *lrp* mutant strain was constructed to gain insight into the role of *lrp* in *Vibrio alginolyticus*. The absence of *lrp* significantly enhances swarming motility, biofilm formation, extracellular protease secretion activity, and tolerance to copper ions. The cumulative mortality of zebrafish (*Danio rerio*) challenged by intraperitoneal injection against the *lrp* mutant strain reached 68.89%, significantly higher than the 40.00% suffered by fish injected with the wild-type strain. The expression levels of *lrp* decreased gradually with increasing culture time under the influence of various physicochemical factors. The expression level of *lrp* was significantly increased after two hours of culture at pH 5, 22°C, 5% NaCl, the presence of 1 mM Cu<sup>2+</sup>, 1/4/7 mM ferric citrate, 0.1 mg/L NaNO<sub>3</sub>, and 0.1 mg/L KH<sub>2</sub>PO<sub>4</sub>. The mRNA level of *lrp* decreased significantly after six hours of culture at 37°C, 1% and 5% NaCl, and the presence of 1/7 mM ferric citrate, 0.1/5 mg/L NaNO<sub>3</sub>, and 0.1/0.5/2.0 mg/L KH<sub>2</sub>PO<sub>4</sub>. The expression of *lrp* increased after ten hours of culture at pH 5/9, 22°C, 1% NaCl, and the presence of 1 mM Cu<sup>2+</sup>, 7 mM ferric citrate, 5 mg/L NaNO<sub>3</sub>, and 0.1 mg/L KH<sub>2</sub>PO<sub>4</sub>. Overall, this study indicates that *lrp* negatively controls the virulence of *V. alginolyticus*, probably by reducing its swarming motility, biofilm formation, extracellular protease secretion activity, and tolerance to copper ions, and that the expression of *lrp* is affected by numerous physicochemical factors, and is especially up-regulated after 2 hours of culture in bacterial growth.

## KEYWORDS

*Vibrio alginolyticus*, *lrp*, biological characteristics, virulence regulation, physicochemical factors

# 1 Introduction

*Vibrio alginolyticus*, a Gram-negative bacterium found throughout the ocean (Ren et al., 2013), has emerged as the primary aquatic pathogenic bacterium in recent years (Li et al., 2021). Diseases caused by *V. alginolyticus* can lead to epidermal bleeding, whitening of the kidneys, and congestion of the internal organs of animals. Its pathogenicity increases significantly in warmer temperatures in summer (Zuo et al., 2019) when it causes severe economic damage to the aquaculture industry (Mohamad et al., 2019; Fahmy and Hamed, 2021; Yang et al., 2021). In humans, *V. alginolyticus* infections can cause septicemia, eardrum infections, and intraocular inflammation (Li et al., 2009; Gaüzère et al., 2016; Baker-Austin et al., 2018). In total, *V. alginolyticus* can not only lead to large-scale mortality in aquatic animals with considerable negative economic impacts, but is also a potential source of human-aquatic animal comorbidity, with a risk to human health. Current studies have shown that the pathogenic effects of *V. alginolyticus* are due to virulence factors such as extracellular products (Wang et al., 2007; Wang et al., 2008; Rui et al., 2009), bacterial adhesion (Wang and Leung, 2000), cell motility (Liu et al., 2020), and biofilm formation (Liu et al., 2011; Liu et al., 2011; Gu et al., 2019b).

Physicochemical factors have an impact on bacterial growth and reproduction. They can also directly influence the expression of bacterial virulence or virulence-regulating genes (DeAngelis et al., 2018), thereby affecting the risks to host organisms (Huang et al., 2015). For example, the abundance of *V. alginolyticus* is affected by factors such as nutrients, temperature, salinity, and phytoplankton (Oberbeckmann et al., 2011). Furthermore, the metabolism of *Vibrio* is significantly influenced by the environmental levels of nitrate, ammonia nitrogen, and phosphate (Unemoto et al., 1977; Oliver et al., 1983; Davis et al., 2017). The formation of *V. alginolyticus* blooms can be promoted by Saharan dust nutrients, which drive changes in its population dynamics (Westrich et al., 2016). Likewise, environmental conditions can substantially impact biofilm formation and adhesion of bacteria (Luo et al., 2016; Toyofuku et al., 2016). For example, the expression of the cytochrome C oxidase gene is effected by temperature, pH, and salinity, which alter the capacity of *V. alginolyticus* to adhere to the host (Huang et al., 2019). *Vibrio alginolyticus* is a conditionally pathogenic bacterium, and external environmental changes can also modulate the expression of pathogenesis-related proteins (Hoe and Goguen, 1993). Outer membrane proteins adapt and respond to environmental stress by altering the expression levels of specific proteins (Abdallah et al., 2012). Environmental factors change periodically both seasonally and geographically. Aquaculture systems are

vulnerable to human activities, including overfeeding, drug usage, industrial pollution, and domestic sewage. The degree of these impacts have changed over time, posing additional challenges and threats, including those which affect the occurrence and development of pathogen virulence (Halpern et al., 2019). Therefore, understanding the influence of environmental factors on bacterial virulence factors or virulence regulators is of great significance in monitoring bacterial virulence and predicting disease occurrence, and can provide a theoretical basis for establishing virulence prevention and control measures based on environmental regulation.

Leucine-responsive regulatory protein (Lrp) belongs to the Leucine-responsive regulatory protein/asparagine synthase C gene product family (Lrp/AsnC family) of globally specific transcriptional regulators, also known as feast/famine regulatory proteins (Brinkman et al., 2003; Yokoyama et al., 2006). Lrp binds regulatory DNA via a helix-turn-helix motif, which strongly influences Lrp-DNA binding (Hart et al., 2011). Lrp primarily responds to exogenous amino acid effectors and globally impacts cellular metabolism (Thaw et al., 2006). Early studies found that Lrp can respond to leucine in *Escherichia coli* (Chen et al., 2001). Further research found that Lrp is involved in the regulation of various metabolic functions, such as amino acid synthesis and catabolism (Brinkman et al., 2003), fimbriae biosynthesis, and pili phase-variation (Nou et al., 1993; Huisman et al., 1994; van der Woude and Low, 1994). As a global regulator, Lrp is associated with cytotoxicity, chemotaxis, and iron uptake in *Vibrio vulnificus* (Ho et al., 2017) and chromosome replication in *Vibrio cholerae* (Ciaccia et al., 2018). Furthermore, numerous studies have shown that Lrp plays a vital role in regulating the virulence of bacteria (Lin et al., 2007; Alice and Crosa, 2012; Hussa et al., 2015; Chen et al., 2019). Lin et al. (2007) found that Lrp could regulate the quorum-sensing regulator AphA, and then activate the expression of the virulence gene. However, in *Vibrio alginolyticus* the *lrp* gene has not yet been the subject of any detailed research, and its function is still unknown.

To understand the function of *lrp* in *V. alginolyticus*, we constructed a null mutant of the *lrp* gene, studied the biological properties and pathogenicity of the  $\Delta lrp$  strain, and then used qRT-PCR to analyze the transcription levels of the *lrp* gene when stressed with different physicochemical factors. These results make a significant contribution to the functional study of *lrp* in *V. alginolyticus*, especially regarding the role of *lrp* in regulating the virulence of *V. alginolyticus* and the effects of physicochemical factors on *lrp* expression. The study will aid the monitoring of *V. alginolyticus* virulence to predict outbreaks of diseases caused by *V. alginolyticus*, as well as provide a theoretical basis for the establishment of the environmental regulation system of *V. alginolyticus* related diseases.

## 2 Materials and methods

### 2.1 Bacterial strains, plasmids, and construction of the *lrp* mutant strain

ZJ-T is a translucent variant of *V. alginolyticus* ZJ-51 (Deng et al., 2016a), and was used as the wild-type strain in this study. ZJ-T cells were cultured in LBS (Luria-Bertani medium with 3% sodium chloride) at 30°C. *E. coli* cells were cultured in LB medium at 37°C. Table 1 lists the bacterial strains and plasmids used in this study.

The *lrp* mutant strain was constructed by homologous recombination (Deng et al., 2016b). The plasmid pSW7848 was linearized with the pair primer pSW7848-F/R, and the two flanking regions of the desired deletion fragments of *lrp* were amplified using the primer pairs dlrp-U-F/R and dlrp-D-F/R, respectively (Table 2). The linearized plasmid and flanking fragments were assembled using a ClonExpress MultiS one-step cloning kit (Vazyme Biotech Co., Ltd., Nanjing, China) with an isothermal assembly. The recombinant plasmid was then introduced into *E. coli* II3813, followed by *E. coli* GEB883. After transformation, the integrity of the flanking fragments was examined using a pair primer Del-check-pSW7848-F/R. After that, the recombinant plasmid was transferred into *V. alginolyticus* ZJ-T by conjugation (Figure 1). The pSW7848 plasmid carries chloramphenicol resistance. D-glucose reduces while L-arabinose induces the expression of the virulence gene *ccdB* in the plasmid (Val et al., 2012). Two conjugation experiments were performed by screening colonies on TCBS plates with either D-glucose or L-arabinose. After conjugation, the mutant strains were obtained by PCR amplification and sequencing verification using the primer pairs Del-*lrp*-check-F/R.

### 2.2 Bacterial growth measurement

The monoclonal clones of *V. alginolyticus* ZJ-T (WT) and the *lrp* mutant strain ( $\Delta lrp$ ) were inoculated into LBS liquid medium and cultured overnight at 30°C with 200 rpm shaking. The overnight cultures were diluted to 0.01 at an optical density of OD600 nm (OD600 nm) in conical flasks and re-cultured at 30°C with 200 rpm continuous shaking. The change of absorbance during 36 h of bacterial growth was measured using a spectrophotometer (INESA Co., Ltd., Shanghai, China). The growth of bacteria in the LBS medium was observed. The experiments were conducted in triplicate, and the results were presented graphically. One-way ANOVA analysis of covariance was performed to analyze the effect of *lrp* deletion with time as a covariate against the OD600 nm values.

### 2.3 Iron absorption, copper ion and H<sub>2</sub>O<sub>2</sub> resistance

The overnight cultures (described in 2.2) were diluted to OD600 nm = 5.0 and then gradient diluted in 10-fold steps (10<sup>0</sup> to 10<sup>9</sup>) with fresh LBS medium. After that, 5  $\mu$ L of each dilution were simultaneously spotted onto LBS plates alone or with either 0.003% H<sub>2</sub>O<sub>2</sub>, 4.5 mM CuSO<sub>4</sub>, or 150  $\mu$ M 2,2'-dipyridyl (DIP). Colony growth was observed after incubation at 30°C for 24 h.

### 2.4 Hemolytic activity

The effect of *lrp* on the hemolytic activity of *V. alginolyticus* was determined using the Mueller-Hinton blood agar plate method

TABLE 1 Strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source
<i>V. alginolyticus</i>		
ZJ-T (WT)	Ap <sup>r</sup> (ampicillin resistant), <i>V. alginolyticus</i> ZJ-T, translucent/smooth variant of wild strain ZJ51, isolated from diseased <i>Epinephelus coioides</i> of the Southern China coast	(Huang et al., 2018b)
$\Delta lrp$	Ap <sup>r</sup> , <i>V. alginolyticus</i> ZJ-T carrying a deletion of <i>lrp</i>	This study
<i>E. coli</i>		
II3813	B462 $\Delta thyA::(erm-pir-116)$ , the intermediate host of suicide vector pSW7848	(Le Roux et al., 2007)
GEB883	<i>E. coli</i> K12 $\Delta dapA::erm pirRP4-2 \Delta recAgyrA462, zei298::Tn10$ ; donor strain of conjugation	(Nguyen et al., 2018)
Plasmids		
pSW7848	Cm <sup>r</sup> (chloramphenicol resistant), Pir protein and <i>ccdB</i> toxin gene are required for replication, suicide vectors with an R6K origin	(Val et al., 2012)
pSW7848- <i>lrp</i> <sup>pup</sup> - <i>lrp</i> <sup>down</sup>	Cm <sup>r</sup> , pSW7848 containing the flanking region fragments in mutant allele of <i>lrp</i>	This study

TABLE 2 PCR primers used in this study.

Primers	Sequences(5'-3')	Application
pSW7848_fwd	GTCTGATTCGTTACCAATTATGACAAC	Amplification of pSW7848
pSW7848_rev	GAATTCGATATCAAGCTTATCGATAC	
dlrp-U-F	ataagcttgatcgaaattcGTCTGTGACAACAAGCGTT	Amplification of upstream fragment for <i>lrp</i> allele exchange
dlrp-U-R	aactgtcactctcgtttggGTAAGGTGGCAGATGGAGATAA	
dlrp-D-F	ccatctgccaccttacCCAAACGAGAGTGACAGTT	Amplification of downstream fragment for <i>lrp</i> allele exchange
dlrp-D-R	taattgtaacgaatcagacTGGATCAACAGGGTCAGT	
Del- <i>lrp</i> -check-F	AAAGCGAACAAACGCCAAAGC	Detection of <i>lrp</i> gene knockout
Del- <i>lrp</i> -check-R	TTGTAAGAGTGCCATCTGCTTTCC	
Del-check-pSW7848-F	TCACTGTCCCTTATTTCGCACC	Detection of recombination
Del-check-pSW7848-R	CTGCTTTTGAGCACTACCCG	
<i>qlrp</i> -F	CAAAGGCTACCGAGCAGTAC	RT-PCR of <i>lrp</i>
<i>qlrp</i> -R	ACATTCACCACCTCGTTTAC	
RT-16S-F	CCTACGGGAGGCAGCAG	RT-PCR reference gene
RT-16S-R	ATTACCGCGGCTGCTGG	

(HuanKai Microbial Sci&Tech. Co., Ltd., Guangdong, China). If the bacteria had caused hemolytic activity, hemolytic circles would appear on the plate. The overnight strain cultures (described in 2.2) were streaked onto Mueller-Hinton blood agar plates and hemolysis was observed after incubation at 30°C for 24 h.

## 2.5 Analysis of swimming and swarming motility

Overnight cultures of the WT and  $\Delta$ *lrp* strains were prepared as described in 2.2. The cultures were diluted with fresh LBS medium to OD<sub>600 nm</sub> = 1.0. Three parallel 5  $\mu$ L samples of the diluted WT and  $\Delta$ *lrp* strains were spotted onto 0.3% agar and 1.5% agar LBS plates and the plates were incubated at 30°C for 16 h and 24 h, respectively. The culture diameters were measured to compare the differences in motility between the WT and  $\Delta$ *lrp* strains.

## 2.6 Analysis of biofilm formation activity

Overnight cultures of the WT and  $\Delta$ *lrp* strains were prepared as described in 2.2, and the bacteria were diluted to OD<sub>600 nm</sub> = 1.0. Samples were pipetted into sterilized 24-well plates with 1 mL per well. The plates were then incubated at 30°C for 48 h and used for biofilm quantification (O'Toole, 2011). After incubation, the wells were aspirated and fixed by adding 10% methanol. Then the biofilms were stained with 1% crystal violet and dissolved in 95% ethanol, followed by measurement of

absorbance values of the biofilms at 550 nm using a microplate reader. The experiment was performed in triplicate and repeated three times.

## 2.7 Extracellular protease secretion assay

In this experiment, the extracellular protease secretion activity of the WT and  $\Delta$ *lrp* strains were determined following Swift et al. (1999) with some modifications. Overnight cultures of the WT and  $\Delta$ *lrp* strains were prepared as described in 2.2. The cultures were diluted to OD<sub>600 nm</sub> = 5.0, and 5  $\mu$ L of the diluted cultures were spotted three times in parallel onto LBS plates containing 1% skimmed milk and then incubated at 30°C. The relative enzyme activity was calculated following Usman et al. (2021). The relative secretion abilities were measured as the diameter of the protein decomposition zone/the diameter of the colony after 24 h.

## 2.8 Antibiotic susceptibility assays

The effect of *lrp* deficiency on the susceptibility of *V. alginolyticus* to 23 antibiotics was determined using the Kirby-Bauer disk diffusion method (Yao et al., 2021). Overnight cultures of the WT and  $\Delta$ *lrp* strains were prepared as described in 2.2. 200  $\mu$ L overnight cultures were mixed with 10 mL fresh LBS liquid medium and poured onto LBS plates. After 10 min wetting, the remaining bacterial solution was poured off. The plates were air-dried, and antibiotic loaded

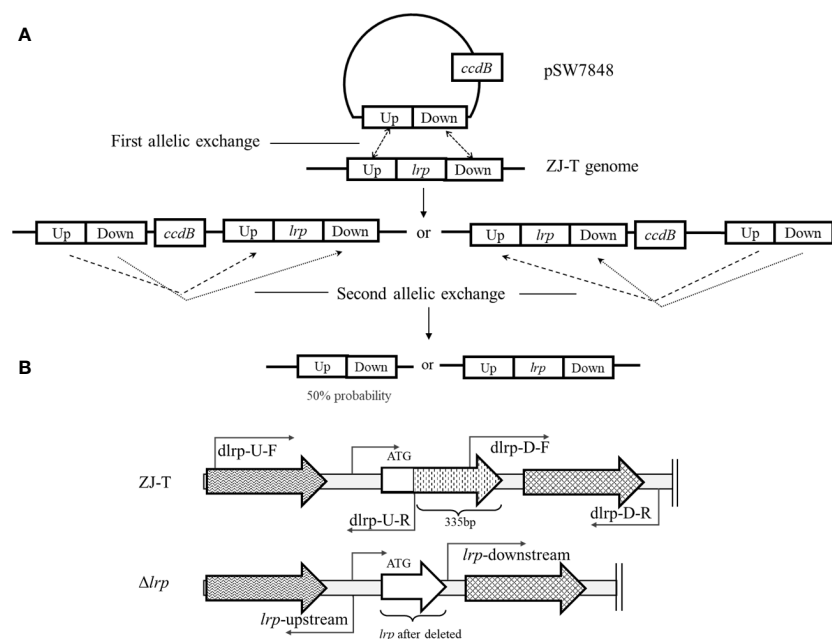


FIGURE 1

The conjugation between recombinant plasmid and *V. alginolyticus* genome (A), and the structure of the *lrp* locus (B). A: TCBS with 0.2% D-glucose and 20  $\mu\text{g}/\text{mL}$  of Cm was used to select transconjugants in the first allelic exchange. TCBS with 0.2% L-arabinose was used to select the transconjugants that underwent plasmid excision in the second allelic exchange. The carried *ccdB* gene expressed was induced by L- arabinose, then pSW7848 was excised. B: 335 bp of the *lrp* ORF were deleted from the 33rd codon to the stop codon, giving rise to the  $\Delta lrp$  strain.

paper pills were placed onto them and then incubated at 30°C. After 24 h, the diameter of the inhibition circle on the plate was recorded, and the sensitivity results were determined by referring to the drug sensitivity instructions.

## 2.9 Zebrafish challenge assay

Zebrafish (average body weight,  $0.35 \pm 0.05$  g) were purchased from an aquarium store in Guangzhou, Guangdong Province and acclimated for two weeks in an indoor laboratory at 30°C with continuous aeration.

Zebrafish challenge experiments were performed to determine the effect of *lrp* on the virulence of *V. alginolyticus*. The WT and  $\Delta lrp$  monoclonal strains were inoculated into LBS agar slant culture medium, then incubated overnight at 30°C. The colonies were then eluted with 3 mL of 0.85% sterile saline, and the bacterial concentration was determined and diluted to OD600 nm = 0.3. The zebrafish were challenged by intraperitoneal injection with 40  $\mu\text{L}/\text{fish}$  ( $2.55\sim 2.71 \times 10^8$  CFU/fish). Each strain was injected into 60 fish equally divided into three tanks. The negative control was injected with 40  $\mu\text{L}/\text{fish}$  of 0.85% sterile saline. Mortality was monitored every 24 h following the challenge, and the cumulative mortalities of four days (96 h) were calculated.

## 2.10 Effect of physicochemical factors on the expression of *lrp* during growth

The overnight cultures of the WT strain was diluted to OD600 nm = 0.01 in LBS medium with different pH (pH 5, 7, and 9), temperature (22°C, 30°C, and 37°C), salinity (1%, 3%, and 5% sodium chloride), copper ions (0.1 mg/L, 1.0 mg/L, and 5.0 mg/L copper sulfate), iron ions (1 mM, 4 mM, and 7 mM ferric citrate), nitrate (0.1 mg/L, 0.5 mg/L, and 2.0 mg/L sodium nitrate), and phosphate (1 mM, 4 mM, and 7 mM potassium dihydrogen phosphate) levels. The pH group, salinity group, and the groups with different concentration of added copper sulfate, ferric citrate, sodium nitrate, and potassium dihydrogen phosphate were cultured at 30°C with 200 rpm shaking. The temperature group were cultured at the different test temperature conditions with 200 rpm shaking. Bacteria cultured in LBS medium at pH 7, 30°C, and 3% NaCl were considered as the controls for the copper ions group, iron ions group, nitrate group, and phosphate group (the pH group use pH 7 as control, temperature group use 30°C as control, and salinity group use 3% NaCl as control). The *lrp* transcription levels of the bacteria cultured in the various conditions were analyzed after 2, 6, and 10 h of growth. RNAiso Plus reagent (Takara Bio Inc., Beijing, China) was used to extract total RNA from the cultures. Reverse transcription was performed using an

Evo M-MLV Mix Kit with gDNA Clean (Accurate Biology Co., Ltd., Hunan, China). RT-PCR amplification was performed to assess the expression of *lrp*, using a SYBR<sup>®</sup> Green Premix Pro Taq HS qPCR kit (Accurate Biology Co., Ltd., Hunan, China). The 16S rRNA gene was used as a control in the qRT-PCR. The primers used for RT-PCR are listed in Table 2. Gene expression analysis was performed using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

## 2.11 Statistical analysis

The effects of *lrp* deletion on the phenotypes and virulence of *V. alginolyticus* and the effects of the various physicochemical factors on the expression of *lrp* were analyzed using Graphpad Prism 9 (Graphpad Software, San Diego, CA, USA). Mean  $\pm$  SD values was calculated for the growth, motility, biofilm formation, extracellular protease, zebrafish challenge assay, and *lrp* expression experiments. Independent two-sample *t*-tests were used to analyze changes in motility, biofilm formation, and extracellular protease secretion activity following *lrp* deletion. A one-way ANOVA was conducted to assess the growth curves and *lrp* expression level assays in the same conditions at the three different time periods. Differences in cumulative mortalities caused by the WT and  $\Delta$ *lrp* strains were analyzed using the Log-rank (Mantel-Cox) test. *P*-values < 0.05 were considered statistically significant.

## 3 Results

### 3.1 Construction of the *V. alginolyticus* ZJ-T *lrp* mutant strain

The *lrp* deletion strain was constructed using homologous recombination following Deng et al. (2016b) with some modifications. The linearized fragment of plasmid pSW7848 (3309 bp), and the upstream and downstream fragments of *lrp* (1025 bp and 904 bp) were successfully amplified using PCR (Figure 2, lane 1-3). The upstream and downstream fragments were then assembled with the linearized suicide vector pSW7848, and the fusion action was transformed into *E. coli* II3813. The fusion was identified using the primer pairs Del-check-pSW7848-F/R, resulting in a fragment of 2214 bp (Figure 2, lane 4). The resulting recombination plasmid pSW7848-*lrp*<sub>up</sub>-*lrp*<sub>down</sub> was subsequently transformed into *E. coli* GEB883. The allelic exchange was performed after the recombinant plasmid was transferred from *E. coli* GEB883 to *V. alginolyticus* ZJ-T by conjugation. The *lrp* disruptant was finally confirmed by sequencing, and the strain was named  $\Delta$ *lrp* (Figure 2, lane 5-6).

### 3.2 Effect of *lrp* on the growth of *V. alginolyticus* under different conditions

The WT and  $\Delta$ *lrp* strains were cultured in LBS medium. The growth of  $\Delta$ *lrp* did not differ significantly from WT during any

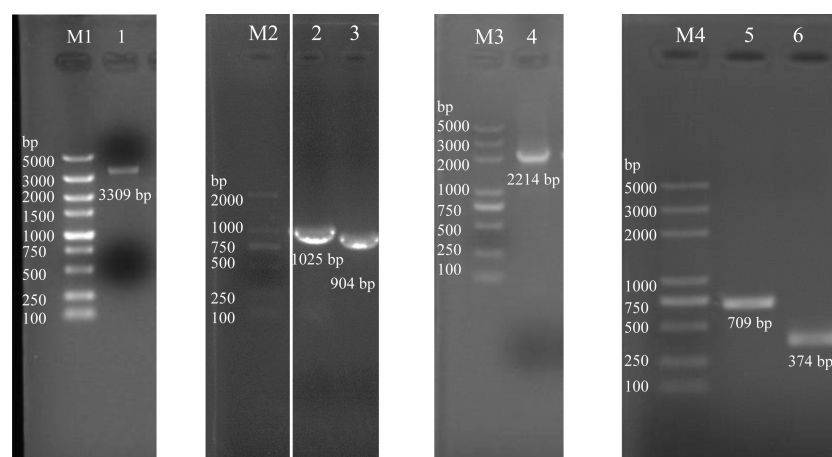


FIGURE 2

PCR identification of  $\Delta$ *lrp* construction in *V. alginolyticus*. M1: 5,000 DNA marker (Guangzhou Dongsheng Biotech Co., Ltd.); M2: 2,000 DNA marker (Guangzhou Dongsheng Biotech Co., Ltd.); M3 and M4: 5,000 DNA marker (Tsingke Biotechnology Co., Ltd.); lane 1: pSW7848 linearized plasmid; lane 2: upstream fragment of *lrp*; lane 3: downstream fragment of *lrp*; lane 4: detecting of the recombination plasmid of pSW7848-*lrp*<sub>up</sub>-*lrp*<sub>down</sub>; lane 5: amplification fragment of wild strain with primers of Del-*lrp*-check-F/R; lane 6: amplification fragment of  $\Delta$ *lrp* with primers of Del-*lrp*-check-F/R. This figure is the combination result of four gel pictures, and they are M1/1, M2/2/3, M3/4, and M4/5/6, respectively. In the gel M1/1, we cut all the lanes on the right of lane 1; in the gel M2/2/3, we cut a lane between lane M2 and lane 2, and cut all the lanes on the right of lane 3, then we spliced the lane M2 and lane 2/3, and there is a space between M2 and lane 2 showing the splicing; in the gel M3/4, we cut all the lanes on the right of lane 4; in the gel M4/5/6, we cut all the lanes on the right of lane 6.

growth period. During the 36 h of incubation, the first hour presented the latent period. The log phase began at 2 h and the stable phase was reached after 12 hours incubation (Figure 3A).

Neither the WT nor  $\Delta lrp$  strains produced hemolytic circles on the Mueller-Hinton blood agar plate (Figure 3B). In the absence of *lrp*, there was no significant difference in the growth of *V. alginolyticus* on either the LBS plate or on the LBS plate with 150  $\mu$ M DIP or with 0.003%  $H_2O_2$  (Figure 3C). As shown in Figure 3C, there was a  $10^5$ -fold difference in survival rate between the WT and the *lrp* mutant strains on the LBS agar plate containing 4.5 mM  $CuSO_4$ . Resistance of *V. alginolyticus* to copper ions was strengthened when *lrp* was absent (Figure 3C).

### 3.3 Absence of *lrp* increased the motility, biofilm formation, and extracellular secretion activity of *V. alginolyticus*

Both the WT and  $\Delta lrp$  strains showed swimming motility on the 0.3% agar LBS plates, but no differences in extension diameter were observed. However, the swarming motility of the  $\Delta lrp$  strain on the 1.5% agar LBS plates was significantly greater than the WT (Figure 4A and Figure 4B).

The biofilm circles were stained with crystal violet and eluted with acetic acid (Figure 4C). The purple biofilm precipitation could be seen at the bottom of the culture wells. The mean amount of  $\Delta lrp$  strain biofilm formed was significantly greater than the WT strain (Figure 4D).

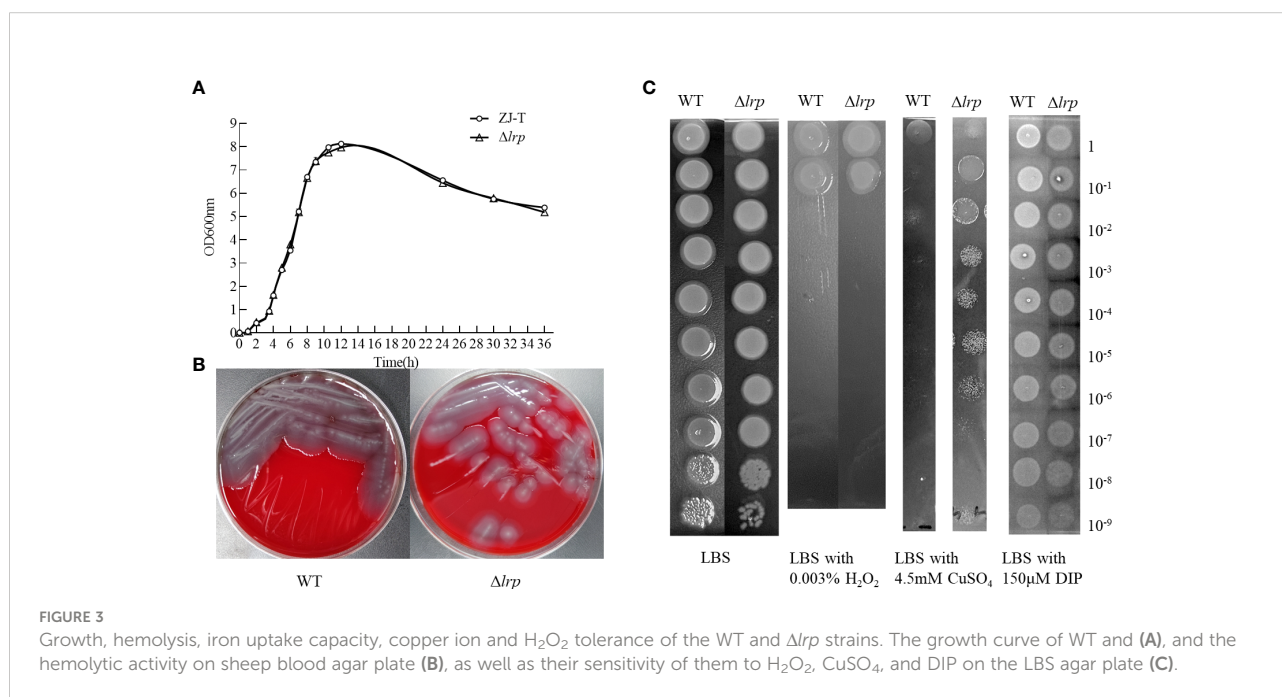
The ratio of the transparent proteolytic circle diameter to the colony diameter indicates the capacity for extracellular protease secretion (Figure 4E). The secretion activity of  $\Delta lrp$  was significantly greater than that of WT (Figure 4F).

### 3.4 No significant effect of *lrp* on antibiotic resistance of *V. alginolyticus*

The bacterial inhibition circles produced around the 23 antibiotic paper tablets revealed that the absence of *lrp* did not significantly change the antibiotic resistance of *V. alginolyticus*. The results showed that the WT and  $\Delta lrp$  strains were resistant to 13 antibiotics, including rifampicin, amoxicillin, sulfafurazole, medimycin, cephalixin, enrofloxacin, furazolidone, compound sulfamethoxazol, streptomycin, cefazolin, tetracycline, ampicillin, and vancomycin. In addition, the absence of *lrp* did not alter the susceptibility of *V. alginolyticus* to 10 antibiotics, including claricid, doxycycline, florfenicol, tobramycin, ciprofloxacin, gentamicin, erythromycin, chloramphenicol, ofloxacin, and norfloxacin (Table S1).

### 3.5 Enhanced virulence of the *lrp* mutant strain to zebrafish

The WT and  $\Delta lrp$  strains were used to challenge experimental zebrafish. The cumulative zebrafish mortality rates in the WT group were 31.11%, 37.78%, 40.00%, and 40.00% for 24 h, 48 h, 72 h, and 96 h after intraperitoneal injection, respectively. The mortality of zebrafish after injection with  $\Delta lrp$  appeared to be greater than for the WT, with cumulative mortality rates of 64.44%, 66.67%, 68.89%, and 68.89% for 24 h, 48 h, 72 h, and 96 h after intraperitoneal injection, respectively (Figure 5). The mortality difference between the WT and  $\Delta lrp$  groups was statistically significant according to a Log Rank (Mantel-Cox) analysis. The results of



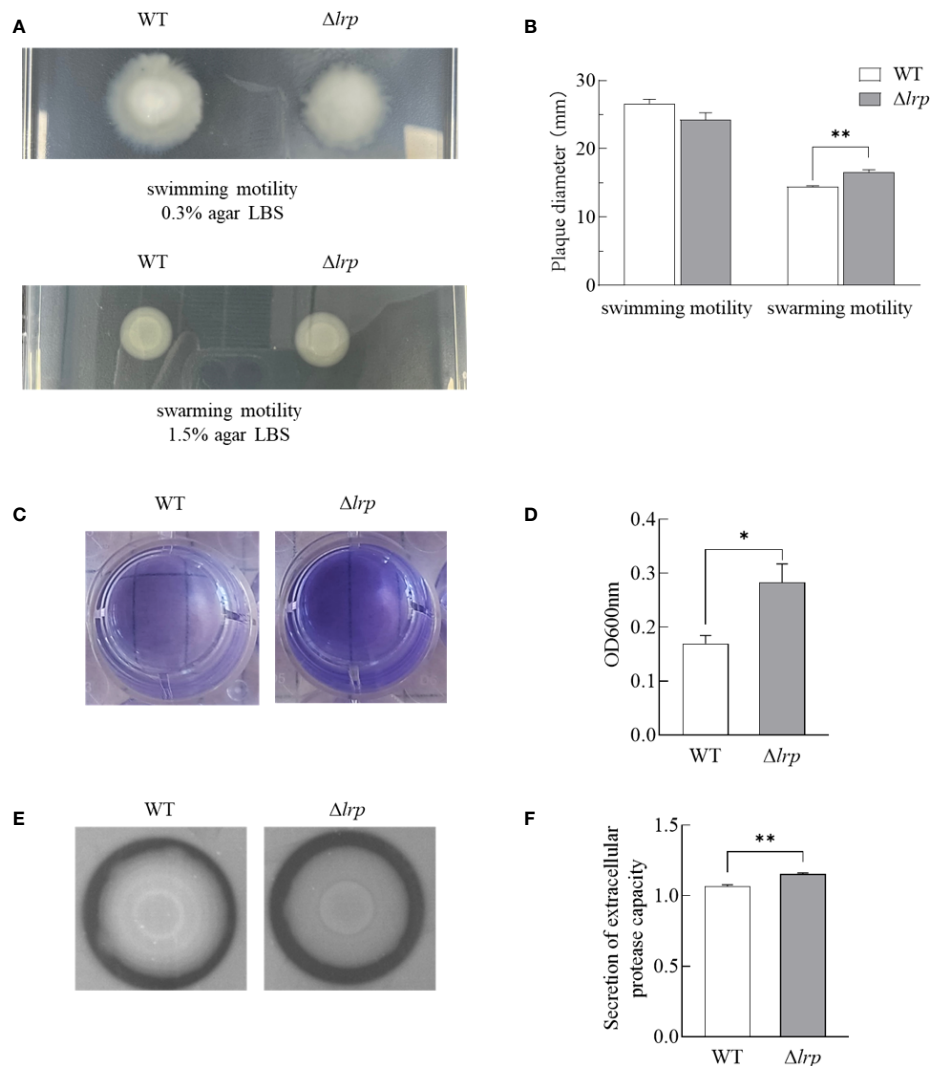


FIGURE 4

Motility, biofilm formation and extracellular protease secretion activity of WT and  $\Delta lrp$  strains. (A): the growth of the strains on the 0.3% and 1.5% agar LBS plate; (B): the colony diameter statistical analysis; (C): dissolved crystal violet-stained biofilm; (D): the absorbance value of biofilm staining; (E): strains growth on the skim milk plate; (F): graph of extracellular protease secretion capacity, vertical coordinate is the ratio of outer circle diameter to inner circle diameter. (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).

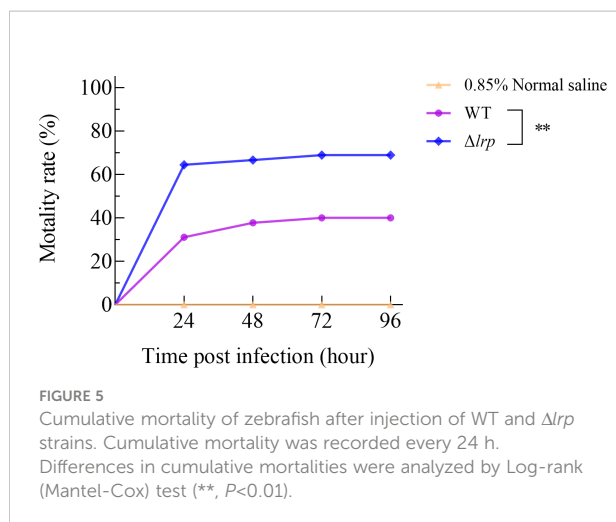
the challenge experiment showed that the deletion of *lrp* enhanced the virulence of *V. alginolyticus* in zebrafish, and that *lrp* may negatively regulate *V. alginolyticus* virulence.

### 3.6 The expression of *lrp* was impacted by various physicochemical factors and was gradually downregulated

In general, the expression of *lrp* showed a gradual decrease with increasing growth under the same cultural conditions. Compared with the control group (pH 7), the expression of *lrp*

increased at pH 5 after 2 h (3.43-fold,  $P < 0.001$ ) and 10 h (1.59-fold,  $P < 0.001$ ) (Figure 6A). At the same incubation time, *lrp* was expressed most at pH 5, followed by pH 7 and pH 9. Meanwhile, the pH 9 group showed a higher *lrp* expression level ( $P < 0.01$ ) at 10 h (Figure 6A). The mRNA of *lrp* increased at a temperature of 22°C at 2 h (3.47-fold,  $P < 0.01$ ) and 10 h (1.52-fold,  $P < 0.05$ ) but decreased at a temperature of 37°C at 2 h (1.75-fold,  $P < 0.05$ ); at 6 h (3.84-fold,  $P < 0.001$ ); and at 10 h (2.13-fold,  $P < 0.001$ ). Culture conditions at a temperature of 37°C could inhibit the expression of *lrp* (Figure 6B). The expression of *lrp* was significantly increased in the 5% NaCl group at 2 h (1.78-fold,  $P < 0.01$ ), but significantly decreased at 6 h ( $P < 0.05$ ) and 10 h





( $P < 0.01$ ). In the 1% NaCl group, its expression was significantly decreased at 6 h (2.63-fold,  $P < 0.001$ ) but increased at 10 h ( $P < 0.05$ ) (Figure 6C).

*lrp* mRNA was generally induced by  $\text{CuSO}_4$ . The 1 mM  $\text{CuSO}_4$  group had a significantly higher *lrp* mRNA expression than the control when cultured for 2 h and 10 h (1.86-fold and 1.36-fold,  $P < 0.01$ , respectively). The expression of *lrp* increased at 6 h when the strain was grown in 3 mM  $\text{CuSO}_4$  (1.49-fold,  $P < 0.01$ ) (Figure 6D). The bacterial cultures with added  $\text{Fe}^{3+}$  (1 mM, 4 mM, and 7 mM) showed relatively higher expression at 2 h compared with the control group, with 4.46-fold ( $P < 0.01$ ), 1.69-fold ( $P < 0.01$ ), and 1.87-fold ( $P < 0.05$ ) increases, respectively. Meanwhile, under the same conditions, *lrp* was usually most expressed at 2 h (Figure 6E). The expression of *lrp* with the addition of 1 mM  $\text{Fe}^{3+}$  and 7 mM  $\text{Fe}^{3+}$  were significantly lower than the expression of *lrp* at 6 h ( $P < 0.01$ ). The 7 mM  $\text{Fe}^{3+}$  group increased *lrp* transcription at 10 h (2.03-fold,  $P < 0.01$ ) (Figure 6E). With the same concentration of  $\text{NaNO}_3$ , the expression of *lrp* was greatest at 2 h, and was significantly increased by the addition of 0.1 mg/L  $\text{NaNO}_3$  (2.07-fold,  $P < 0.001$ ). Appropriate concentrations of  $\text{NaNO}_3$  decreased *lrp* expression at 6 h (0.1/5.0 mg/L  $\text{NaNO}_3$ ,  $P < 0.05$ ), while the expression of *lrp* was increased at 10 h in 5.0 mg/L  $\text{NaNO}_3$  (1.59-fold,  $P < 0.001$ ) (Figure 6F). The *lrp* expression of WT in LBS generally decreased with increasing concentrations of  $\text{KH}_2\text{PO}_4$ , except that the 0.1 mg/L  $\text{KH}_2\text{PO}_4$  group significantly increased *lrp* expression at 2 h (1.55-fold,  $P < 0.05$ ) and 10 h (2.10-fold,  $P < 0.001$ ). The *lrp* expression of the 0.5 mg/L  $\text{PO}_4^{3-}$  and 2.0 mg/L  $\text{PO}_4^{3-}$  groups decreased at 2 h and 6 h, respectively (Figure 6G).

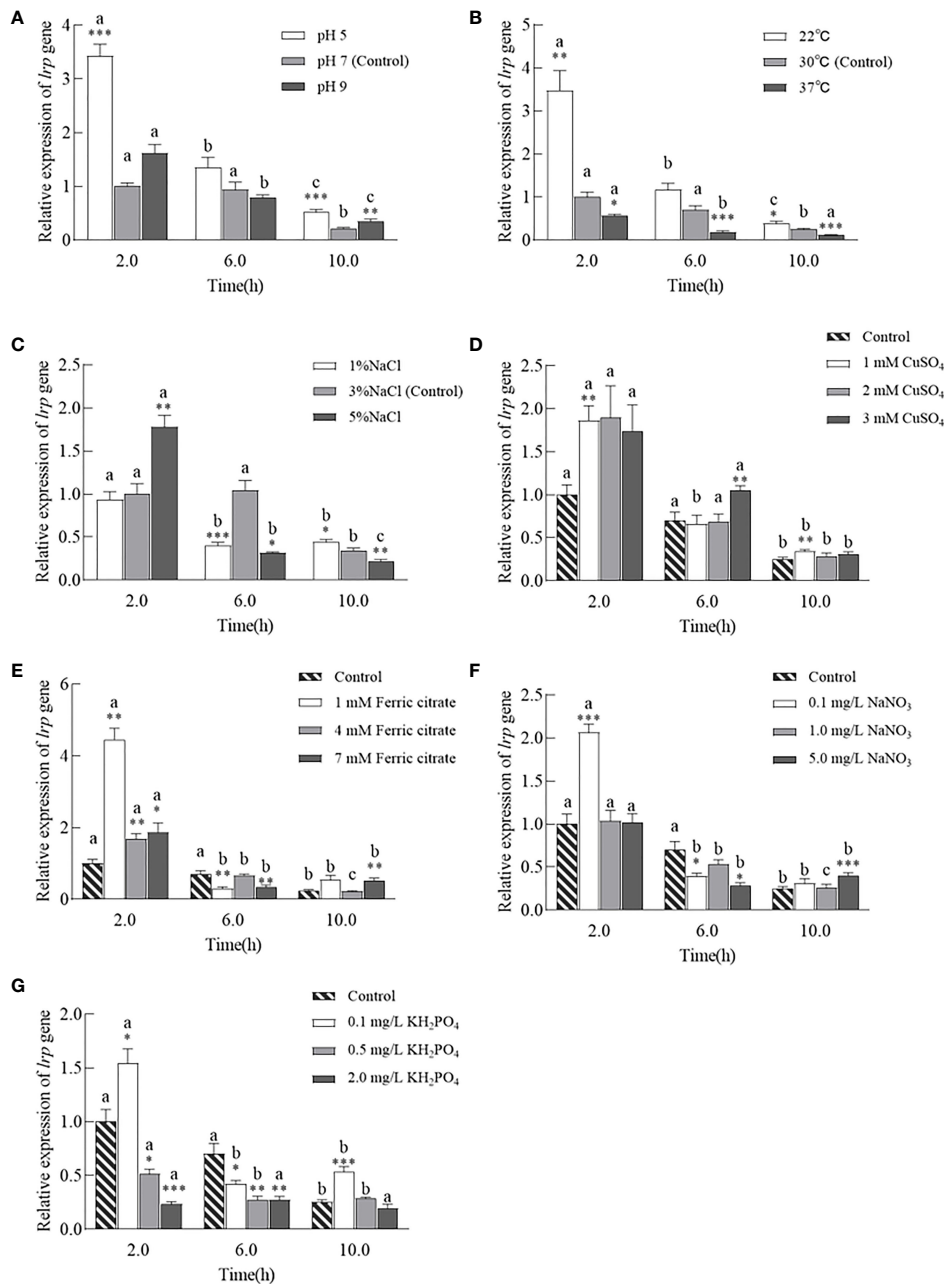
## 4 Discussion

In this study, a *lrp* mutant strain of *V. alginolyticus* was constructed to explore the role of Lrp and its biological properties and virulence. Biophenotype characterization revealed that the

deletion of *lrp* did not significantly affect growth, iron absorption, radical oxygen resistance, hemolytic activity, antibiotic resistance, or swimming motility but increased swarming motility, biofilm formation, secretion of extracellular protease, and copper ion resistance. The virulence of *V. alginolyticus* to zebrafish was significantly enhanced after *lrp* deletion. *lrp* expression was affected by many physicochemical factors, including pH, temperature, salinity, and the presence of copper sulfate, ferric citrate, sodium nitrate, and potassium dihydrogen phosphate.

Bacteria move in highly viscous environments using their flagella, which help them to invade and colonize their hosts (McCarter and Silverman, 1990; Josenhans and Suerbaum, 2002). Many genes have been associated with bacterial motility (Yuan et al., 2022). Liu et al. (2021) showed that the RNA binding protein, CsrA, activates swarming motility by enhancing the expression of lateral flagellum-related genes. Deleting the quorum sensing-regulated extracellular protein gene *pep* leads to reduced motility and failure to generate lateral flagella (Cao et al., 2011). Biofilm formation is encouraged by bacterial spores and flagella (Yildiz and Visick, 2009) and helps bacteria to colonize of the host's interior or the surface of aquatic plankton (Tamplin et al., 1990). Motility therefore plays a crucial role in biofilm formation, pathogenicity, and environmental adaptation in many bacteria (Gu et al., 2019a). The lateral flagella support bacterial swarming motility over surfaces or through viscous environments (McCarter, 2004). Gu et al. (2019a) reported that a GntR family transcription factor may increase swarming motility by enhancing the transcription of lateral flagella genes in *Vibrio parahaemolyticus*. The flagellin-homologous proteins (FHPs) in *V. vulnificus* could enhance the exopolysaccharide-enriched biofilm matrix through excretion to the extracellular milieu. Therefore, the presence of FHPs involved in biofilm enhancement could promote the pathogenicity of bacteria (Jung et al., 2019). In this study, bacterial swimming motility was not affected by *lrp* deletion, while swarming motility was significantly increased. The absence of *lrp* may induce the flagella to move when crossing surfaces or in sticky environments. Lrp may affect biofilm formation and swarming motility by regulating FHPs secreted into the extracellular environment. Changes in biofilm formation and cell motility may lead to enhanced virulence of *V. alginolyticus* in zebrafish. Therefore, because of the changes in motility and biofilm formation after *lrp* deletion found in this study, we hypothesize that *lrp* may affect the expression of the flagella gene in *V. alginolyticus* to regulate its swarming motility, and so influence biofilm formation and lead to changes in virulence.

Extracellular protease secretion activity also increased after deletion of the *lrp* gene. Extracellular proteases are pathogenic factors and play an essential role in the infection process of *V. alginolyticus* (Watnick et al., 2001). Extracellular production of the alkaline serine protease has been reported to be lethal to fish (Lee et al., 1997), and the alkaline serine protease gene *asp* is regulated by the quorum sensing system, resulting in changes in



**FIGURE 6**  
 Expression profiles of WT strains under 7 incubate conditions. Expression levels of strains cultured in LBS with different pH (A), temperature (B), salinity (C), and addition of different concentrations of copper ion (D), iron ion (E), nitrate (F), phosphate (G). Data are expressed as means  $\pm$  SE relative to control group. Significant differences between control group and treatment groups at the same growth period are indicated with asterisks (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). Take strains with the same culture conditions as a group and use different letters to indicate significant differences in the growth period of the same group ( $P < 0.05$ ).

virulence (Rui et al., 2009). The response regulator protein LuxO has multiple roles in regulating extracellular products, and the extracellular protease activity was significantly enhanced after *luxO* deletion (Wang et al., 2007). The results of this study are similar to those described above. After *lrp* mutation, extracellular

protease secretory activity was significantly increased, as were swarming motility and biofilm formation. Further research is needed to determine whether Lrp can regulate virulence by inducing changes in extracellular protease activity, and interact with biofilm formation or flagellar gene expression.

Copper, in its two oxidation states, is involved in essential redox reactions and promotes the formation of reactive oxygen species, leading to cellular damage (Karlin, 1993). Copper efflux is essential to bacterial pathogens for host colonization and virulence and is primary prerequisite for *Vibrio* intracellular phases and cytotoxicity to hemocytes (Kong et al., 2015; Vanhove et al., 2016). Kong et al. (2015) found that the adhesion capacity of *V. alginolyticus* was significantly reduced after treatment with copper ions (50 mg/L). In *V. cholerae*, copper resistance proteins were reduced in *nqr* (electrogenic, sodium ion-translocating NADH: quinone oxidoreductase gene) deletion strains, resulting in diminished copper resistance (Toulouse et al., 2018). Vanhove et al. (2016) found that the primary mechanism of copper ion resistance was to prevent the host phagocytes killing pathogens, and to induce the cytolysis of host immune cells and host colonization. In this study, the mutant strain  $\Delta lrp$  was less damaged by copper ion stress than the WT, indicating that its sensitivity to copper ions was reduced after the mutation ameliorated the toxicity of copper ions. It appears that Lrp may regulate the expression of copper resistance proteins in *V. alginolyticus*, decreasing its sensitivity to copper ions and enhancing its adhesion capacity.

Physicochemical factors can influence bacterial gene expression, thus regulating the biological properties relating to virulence. The attachment of *V. alginolyticus* was affected by temperature, which changed the expression of *rstA* and *rstB*. These two genes have a regulatory role in bacterial adhesion, biofilm production, motility, and hemolysis (Huang et al., 2018a). Appropriate salinity levels facilitated the adherence of *V. alginolyticus* to the skin mucus of *Sparus aurata*, and *V. alginolyticus* showed a higher adhesion rate in seawater (3.5% salinity) than in PBS and saline solution (Balebona et al., 1995). Studies have shown that the adhesion ability of *V. alginolyticus* was impaired under heavy metal (50 mg/L  $\text{Cu}^{2+}$ , 100 mg/L  $\text{Pb}^{2+}$ , 50 mg/L  $\text{Hg}^{2+}$ ) and acidic stresses (Kong et al., 2015). VspR is the primary regulator in *V. alginolyticus*, and enables the formation/maintenance of biofilms by directly sensing phosphate levels (Hsieh et al., 2022). In this study, the expression of *lrp* was generally up-regulated by different physicochemical factors, and *lrp* usually showed its highest expression after 2 h of culture time. Many combinations of adverse environmental stresses could increase the expression of *lrp* (pH 5, 22°C, 5% NaCl, 1/4/7 mM  $\text{Fe}^{3+}$ , 0.1/5 mg/L  $\text{NO}_3^-$  and 0.1 mg/L  $\text{PO}_4^{3-}$  at 2 h; and pH 5, pH 9, 22°C, 1% NaCl, 1 mM  $\text{Cu}^{2+}$ , 7 mM  $\text{Fe}^{3+}$ , 5 mg/L  $\text{NO}_3^-$ , and 0.1 mg/L  $\text{PO}_4^{3-}$  at 10 h). The virulence of *V. alginolyticus* was reduced in the presence of *lrp*, indicating that increased *lrp* expression of ZJ-T in different conditions might also decrease its virulence. Some conditions could decrease the transcription level of *lrp* at 6 h, including 37°C, 1% NaCl, 5% NaCl, 1/7 mM iron ions, 0.1/5 mg/L nitrate, 0.1/0.5/2 mg/L phosphate. The significant decrease in *lrp* expression indicated that the virulence of *V. alginolyticus* might increase over a similar time in these culture conditions. These culture

conditions may lead to changes in biological characteristics similar to those found in  $\Delta lrp$ , such as motility, biofilm formation, and enhanced extracellular protease secretion. Therefore, various physicochemical factors that change the expression of *lrp* probably contribute to variations in adhesion, colonization, and toxin release of *V. alginolyticus*, thereby leading to changes in virulence. Especially after 2 h and 10 h of cultivation, the physicochemical factors tested tend to promote *lrp* expression, thereby probably inhibiting the virulence of *V. alginolyticus*. However, whether the increased expression of *lrp* in adverse environments is related to the maintenance of ZJ-T homeostasis or is involved in regulating the secretion of factors related to flagella, clearly requires further study.

## 5 Conclusions

After *lrp* deletion, *V. alginolyticus* displayed dramatically increased virulence, swarming motility, biofilm formation, extracellular protease activity, and resistance to copper ions. The expression of *lrp* showed a gradual decrease with time in any one cultural environment. After 2 h incubation time, cultivation with pH 5, 22°C, 5% NaCl, 1 mM  $\text{Cu}^{2+}$ , 1/4/7 mM  $\text{Fe}^{3+}$ , 0.1 mg/L  $\text{NO}_3^-$ , 0.1 mg/L  $\text{PO}_4^{3-}$  increased *lrp* expression, and at 37°C, 0.5 mg/L  $\text{PO}_4^{3-}$  and 2 mg/L  $\text{PO}_4^{3-}$  *lrp* expression was inhibited. After 6 h of culture at 37°C, 1/5% NaCl, 1/7 mM  $\text{Fe}^{3+}$ , 0.1/5 mg/L  $\text{NO}_3^-$ , 0.1/0.5/2 mg/L  $\text{PO}_4^{3-}$  the expression of *lrp* was inhibited. *lrp* expression was promoted after 10 h of culture at pH 5/9, 22°C, 1% NaCl, 1 mM  $\text{Cu}^{2+}$ , 7 mM  $\text{Fe}^{3+}$ , 5 mg/L  $\text{NO}_3^-$ , and 0.1 mg/L  $\text{PO}_4^{3-}$ , but was inhibited at 37°C and 5% NaCl. Therefore, Lrp was strongly affected by various physicochemical factors and may reduce the virulence of *V. alginolyticus* by inhibiting biofilm formation, swarming motility, and extracellular protease secretion. These results further the study of virulence regulation in *V. alginolyticus*. Future research should focus on: (1) how *lrp* affects motility, biofilm formation, and extracellular protease secretion, and ultimately the virulence of *V. alginolyticus* at the gene regulation level; and (2) the effects of various levels of virulence of *V. alginolyticus* on aquatic animals under different environmental conditions, including temperature, pH, salinity, and the presence of copper sulfate, ferric citrate, sodium nitrate, and potassium dihydrogen phosphate. The results of such work will help to establish precise virulence control strategies based on environmental regulation.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, CP016224.1.

## Ethics statement

The animal study was reviewed and approved by the study protocol was approved by the Animal Care and Use Committee of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences and performed in accordance with institutional regulations and guidelines.

## Author contributions

WS: Completed experiment, Data curation, investigation, software, and writing. YD: Funding acquisition, conceptualization, resources, writing-review, and editing. SZ: Aided experiment conduct and validation, data collation. SG: Visualization, data curation, and validation. JF: Funding acquisition, project administration, supervision, writing-review and editing, validation. All authors contributed to the article and approved the submitted version.

## Funding

This work was supported by the Central Public-Interest Scientific Institution Basal Research Fund, CAFS (2022GH03), the Guangzhou Science and Technology Program (202201010162), the National Natural Science Foundation of China (NSFC) (31902415), Central Public-interest Scientific

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Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (2021SD15), the Natural Science Fund of Guangdong (2019A1515011833), and the China Agriculture Research System (CARS-48).

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.1042899/full#supplementary-material>

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