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ORIGINAL ARTICLE

Cu^{2+} regulated sulfonamides resistance gene (*sul*) via reactive oxygen species induced ArcA in a pathogenic *Vibrio splendidus*

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Abstract

Purpose To detect the sulfonamide resistance of *Vibrio splendidus* strain Vs, and characterize the factors that regulate the expression of sulfonamide resistance gene sul_{Vs}.

Methods Minimal inhibitory concentration was measured using growth inhibition method, gene expression was measured using real-time reverse transcriptase PCR, reactive oxygen species (ROS) production under Cu^{2+} or Cu^{2+}/NAC conditions was measured using an excitation wavelength of 485 nm, and an emission wavelength of 525 nm, and the binding of sul_{Vs} promoter and the transcriptional factor ArcA was measured using electrophoretic mobility shift assay (EMSA).

Result sul_{Vs} gene was cloned and its expression was upregulated to 6.98- and 3.57-fold in the presence of sulfadiazine and sulfamethoxazole, respectively. Moreover, Cu^{2+} could also upregulate the expression of sul_{Vs} to 14.27-fold, the production of ROS to 4.37-fold, and the expressions of antioxidant-related genes and a transcriptional regulator *arcA* gene. After addition of N-Acetyl-L-cysteine, a ROS inhibitor, the production of ROS decreased to 53.1%, and the expression of *arcA* gene was also downregulated to 26%. EMSA showed that the purified recombinant ArcA could directly bind to the promoter region of sul_{Vs} with specificity.

Conclusion *V. splendidus* strain Vs showed sulfonamide resistance due to sul_{Vs}, and Cu^{2+} could increase the level of ROS followed by ArcA activation as a transcriptional factor to increase the expression of sul_{Vs}.

Keywords *Vibrio splendidus* strain Vs · Sulfonamides resistance gene (*sul*) · *arcA* · Reactive oxygen species (ROS)

Introduction

Vibrio splendidus is an opportunistic pathogen that ubiquitously presents in the marine environment. It can infect many hosts including fish, shellfish, and sea cucumber, leading to bacterial diseases (Helen et al. 2009; Liu et al. 2013; Zhang et al. 2016). Synthetic sulfonamides have been widely used to treat bacterial infections for their wide antibacterial spectrum and effective antibacterial action in humans and animals, since it was introduced into clinical practice from 1935. Sulfonamides function via competing with the natural substrate pamino-benzoic acid for dihydropteroate synthase, resulting reduced production of dihydrofolic acid and thus leading to death of bacteria (Stokstad and Jukes 1987). Now, levels of sulfonamides in the environment have reached a moderate pollution risk for their excessive and frequent use, and they have imposed persistent risks to the living organisms (Richardson et al. 2005; Hernando et al. 2006). Environmental bacteria can easily acquire antibiotic resistances after being exposed to residual antibiotics. Beside antibiotics, heavy metals were also widely used to treat bacterial diseases, leading to synergistic co-contamination of antibiotics and heavy metals (Mu et al. 2014; Wu et al. 2015). While abundance of antibiotic resistance genes (ARGs) was closely linked to the levels of residual antibiotics that exerted a selection pressure on microorganisms (McKinney et al. 2010), some reports showed that expression of ARGs showed no or weak correlation with antibiotic levels, but it was strongly correlated with levels of heavy metals (Baker-Austin et al. 2006).

Bacteria could produce reactive oxygen species (ROS), such as O^{2-}, hydrogen peroxide (H_2O_2), hydroxyl radicals ([·OH]),

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nitric oxide (NO), and singlet oxygen ([·O_2]), which are important signaling molecules with strong oxidizing properties (Daniel et al. 2009). These chemicals play important roles in various signal transductions in vivo. When the cells are under heavy metals stress, the redox balance will be broken, and ROS will be generated in large quantities, and thus leading to oxidative stress (Sies 1997; James and Christopcher 2010). As a consequence, living organisms have developed enzymatic and non-enzymatic antioxidant defense systems to balance the excessive ROS. Among different kinds of ROS, O^{2-} was one of the most important species generated from NADPH by a typical environmental sensitive membrane protein (Han et al. 2012; Radermacher et al. 2012). Transcriptional regulator ArcA plays an important role in maintaining the ratio of NADH to NAD^+ to avoid excessive production of ROS (Loui et al. 2009).

In the present study, the effects of Cu^{2+} on the expression of sul_{Vs} gene, ROS production, and expression of *arcA* gene were determined in *V. splendidus*. The relationship between ROS production and expressions of *arcA* and sul_{Vs} was examined. Based on the data obtained, a regulation pathway of Cu^{2+} on expression of sul_{Vs} was preliminarily proposed.

Materials and methods

Bacterial strains, culture conditions, and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani Lysis Broth (LB) at 37 °C. *V. splendidus* strain Vs was isolated from the diseased *Apostichopus japonicus* (Zhang et al. 2016), and was grown in 2216E medium (5 g tryptone, 1 g yeast extract, and 0.01 g $FePO_4$ in 1 L aged seawater) at 28 °C. Ampicillin (Amp), kanamycin (Kan), or X-gal (5-bromo-4-chloro-3-indolyl-beta-Dgalactopyranoside) was separately supplemented at concentrations of 50, 50, or 40 μg/ml, when it was needed.

Cell density was measured at 600 nm using an UV–Vis (Molecular Device, USA). N-Acetyl-L-cysteine (NAC) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Electrophoretic mobility shift assay (EMSA) Gel-shift binding buffer (5×) was purchased from Beyotime (Shanghai, China). 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma-Aldrich. Taq and Pfu DNA polymerases were purchased from Takara (China). Restriction endonucleases were purchased from New England Biolabs and used in accordance with the manufacturer's specifications. Unless otherwise stated, all other chemicals used in this study were of the highest purity and were purchased from Sangon (Shanghai, China).

Plasmid construction

Plasmid extraction, DNA extraction from agarose gels, and the purification of PCR products were performed using the respective kits from Omega Bio-Tek (GA) according to the manufacturer's instructions. The primers used in this study are listed in Table 2. DNA sequencing was carried out by Sangon (Shanghai, China). pET28a-*arcA* was constructed by ligating *arcA*, amplified with the forward primer arcAF3 and reverse primer arcAR3, into pET-28a, between the *BamH* I / *Xho* I sites. To construct pSC11-$Psul_{Vs}$, $Psul_{Vs}$ was amplified by PCR using Pfu DNA polymerase with primers of P3F6 and P3R6. The PCR product was phosphorylated using T4 polynucleotide kinase, and then was inserted into the *Swa* I site of pSC11, a low-copy-number plasmid with a pSC100 replication origin and a promoterless *lacZ* gene as a reporter of heterologous promoter activity (Zhang et al. 2008).

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
V. splendidus strain Vs	Amp^R: *A. japonicus* pathogen	Zhang et al. (2016)
E. coli		
DH5α	Host strains for general cloning	TaKaRa (China)
BL21 (DE3)	Host strains for gene expression	TaKaRa (China)
Plasmids		
pET-28(a)	Kan^R; general expression vector	Novagen (United States)
pET28a-*arcA*	Kan^R; pET28a expression *arcA*	This study
pSC11	Kan^R; Promoter probe plasmid	Zhang et al. (2008)
pSC11-P1	Kan^R; pSC11 carrying P1	This study
pSC11-P2	Kan^R; pSC11 carrying P2	This study
pSC11-P3	Kan^R; pSC11 carrying P3	This study

Table 2 Primers used in this study

Primer	Sequences (5′ - 3′)*
P1F1	TTGCGAACCTTGTTAACGAAGCGGC
P1R1	CGAGCAATATCTGTTGCACGTTCG
P3F6	CCAAAAGCTGAAGAGTCGAAAG
P3R6	GGAAGGCTGGTCTTAAAAGGTT
arcAF3	GGATCCATGCAAACCCCGCAGATTCTTATCG (*BamH* I)
arcAR3	CTCGAGTTAGTCTTCTAGGTCACCACAAAAG (*Xho* I)
arcAdl F4	CACTAAGCCTTTCAACCCTC
arcAdl R4	GAAGTGAAGTAGAGCACGGAAC
FtF1	GCGCCAAAACATGAATTCGAC
FtR1	CTGCAGGAGCTTCCATAATTG
NoxF1	CTGGGCGAACGGTGGAAT
NoxR1	GGGATCTGTTTGCCTGGGTG
SODF1	CTGTCTTTACGCCGGAATTG
SODR1	CCATTACTGGTTGGTCAGCTAC
SoxRF1	GCCTTCGGCTCTGCGTTTCT
SoxRR1	GCCCTTGCCACTTCTTAGCG
933F	GCACAAGCGGTGGAGCATGTGG
16SRTR1	CGTGTGTAGCCCTGGTCGTA

*Underlined nucleotides are restriction sites of the enzymes indicated in parentheses

Expression and purification of the recombinant ArcA

E. coli BL21(DE3) harboring the plasmid pET28a-*arcA* was grown to mid-log phase, and expression of the recombinant ArcA was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 μM. After grown for an additional 6 h, the cells were harvested by centrifugation and the His-tagged ArcA was purified with nickel-nitrilotriacetic acid agarose under native conditions according to the recommendations of the manufacturer (QIAGEN, Valencia, CA) (Zhang and Sun 2007).

EMSA

EMSA was carried out according to the previous description by Wang et al. (2008). Fifty nanograms of $Psul_{Vs}$ or negative control DNA amplified using the primers P3F6/P3R6 or P1F1/P1R1 was separately mixed with various amounts of purified recombinant ArcA, EMSA/Gel-shift binding buffer (5×) and the nuclease-free water were added to make up the volume of 10 μl, and incubated at 25 °C for 45 min. The samples were then electrophoresed in a non-denatured 8% polyacrylamide gel in an ice bath, and the gel was dyed with nucleic acid dye after electrophoresis for 1 h. Observation was conducted under a gel imaging system (Junyi, China).

ROS measurement

V. splendidus strain Vs was cultured in 2216E medium to approximately OD_{600} of 0.2, and then it was departed into two parts. One part was added with 0.5 mM Cu^{2+}, and the other part without any addition was used as a control. Cell precipitates were obtained from the culture at a time intervals of 15, 30, and 45 min by centrifugation at 6000*g* for 5 min. To quantify ROS production after sole NAC or the combined treatment of NAC and Cu^{2+}, *V. splendidus* strain Vs was cultured in 2216E medium to approximately OD_{600} of 0.2, and then it was departed into three parts. One millimolar NAC, or both 0.5 mM Cu^{2+} and 1 mM NAC was added into separate part and one part without addition was used as a control. The cell pellet was washed three times with PBS (1×), and then it was resuspended in PBS (1×) to an OD_{600} of 0.1. DCFH-DA was used to treat samples, followed by the quantitation of intracellular ROS production. ROS was determined according to the protocol of Varioskan™, and fluorescence was detected using an excitation wavelength of 485 nm, and an emission wavelength of 525 nm (Thermo Fisher Scientific, MA).

Sample collection for real-time reverse transcriptase PCR (RT-PCR)

To detect the effects of SDZ and SMZ on the expression of sul_{Vs}, *V. splendidus* strain Vs was cultured in 2216E medium to approximately OD_{600} of 0.2, then cells were divided into three aliquots. One aliquot was supplemented with 64 μg/ml

SDZ, the second aliquot was supplemented with 32 μg/ml SMZ, and the third aliquot was continuously cultured in medium without addition of SDZ or SMZ. All the three aliquots were cultured for another 10, 20, and 30 min.

To determine the effect of Cu^{2+} on the expression of *arcA* and other antioxidant-related genes, *V. splendidus* strain Vs was grown in 2216E medium to OD_{600} of approximately 0.2, and then it was departed into two parts. One part was supplemented with 0.5 mM Cu^{2+}, and the other part without any addition was used as a control. Then, each sample was cultured for another 30 and 45 min. To detect the effects of simultaneous presence of Cu^{2+} and NAC on the expression of *arcA*, *V. splendidus* strain Vs was grown in 2216E medium to OD_{600} of approximately 0.2, then cells were divided into three aliquots. One aliquot was supplemented with 1 mM NAC, the second aliquot was simultaneously supplemented with 0.5 mM Cu^{2+} and 1 mM NAC, respectively, and the third aliquot without any addition was used as a control. Then, each sample was cultured for another 30 and 45 min.

After treated under the above conditions, all of the cells were quickly freezed in − 80 °C pre-incubated ethanol, and then centrifuged to collect the pellets. The cell pellets were then resuspended in RNAlater, and then centrifuged again to collect the pellets. Then, the cell pellets were used for RNA extraction.

Real-time RT-PCR

Total RNA was extracted using the bacterial RNA kit (Omega), followed by the treatment of RNase-free DNase (Promega, USA) for 1 h. The DNA-free RNAs were used for the synthesis of cDNA using the reverse transcriptase (Taraka, China). Real-time RT-PCR was carried out as described by Zhang et al. (2013b). Real-time RT-PCR was carried out in an ABI 7500 real-time detection system (Applied Biosystems) by using the SYBR ExScript RT-PCR kit (Takara, China). The primers for *arcA* and antioxidant-related genes were arcAdl F4/arcAdl R4, FtF1/R1, SODF1/R1, NoxF1/R1, and SoxRF1/R1. Each assay was performed in triplicate using 16S rRNA as a control. The primers used for 16S rRNA were 933F and 16SRTR1. The comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative mRNA levels of *arcA* and antioxidant-related genes.

Database search and data analysis

Searches for nucleotide and amino acid sequence similarities were conducted using the BLAST programs from the NCBI (National Center for Biotechnology Information). Isolated of *V. splendidus* strain Vs has been deposited into the China General Microbiological Culture Collection (CGMCC, Beijing, China) with accession numbers CGMCC No. 7.242. The homologous analysis of amino acid sequence was analyzed by MEGA 5.1 (Tamura et al. 2007) and CLUSTAL X (Larkin et al. 2007). Bacterial promoter prediction was performed using BPROM (http://www.softberry.com). Statistical analyses were performed by using the two-tailed Student *t* test. One-way ANOVA was performed to determine the significant differences between experimental and control groups at each specific time point, and the significance level was defined as *, $P < 0.05$, and **, $P < 0.01$.

Results

V. splendidus showed resistance to sulfonamides via sul_{Vs}

The MICs of SDZ, SMZ, and TMP are listed in Table 3. The inhibitory effect of SDZ on growth of *V. splendidus* strain Vs was dose dependent. *V. splendidus* strain Vs showed obvious growth in medium supplemented with 512 μg/ml SDZ, but showed no obvious growth in medium supplemented with 1024 μg/ml SDZ. Thus, the MIC of SDZ to *V. splendidus* strain Vs was 1024 μg/ml under the test conditions. We have sequenced the whole genomic DNA of *V. splendidus* strain Vs (unpublished data); one dihydropteroate synthase gene was annotated and it showed 88.69% homolog to the nucleotide sequence of dihydropteroate synthase gene from the published chromosome of *V. splendidus* strain LGP32 (No. FM954972). Nucleotide sequence analysis showed that ORF was 831 bp and coded dihydropteroate synthase with an estimated molecular mass of 30.06 kDa and theoretical pI was 5.12. The homology analysis showed that the dihydropteroate synthase gene of *V. splendidus* showed best homology to sulfonamide resistance gene (Fig. 1a), and thus, it was named sul_{Vs}. Comparison with the known sequence data of sul_s indicated that the best matches for the sul_{Vs} were the *suls* from *Bacillus amyloliquefaciens* (CP000560.1), and *Thermoanaerobacter kivui* (AIS53244.1), which showed, 43.63% and 42.91% identity to the amino acid sequence of sul_{Vs}, respectively. The expression of sul_{Vs} significantly was upregulated to 6.98-fold and 3.57-fold in the presence of SDZ and SMZ (Fig. 1b).

Cu^{2+} induced ROS and expressions of sul_{Vs} and *arcA* genes

Expression of sul_{Vs} could be upregulated to 14.27-fold in the presence of Cu^{2+}. Beside that, Cu^{2+} also induced the production of ROS and the expression of *arcA* gene. ROS increased 2.85-, 4.37-, and 2.54-fold at 15, 30, and 45 min, respectively, after the addition of Cu^{2+} (Fig. 2). The mRNA levels of antioxidant-related genes also correspondingly increased at 30 and 45 min, respectively, and the highest stimulatory effect was obtained in the cells collected at 45 min (Fig. 3a–d). Taken all these results together, it proved that Cu^{2+} could

Table 3 MICs of antibiotics to *V. splendidus* strain Vs

Antibiotics	MIC (μg/ml)	Performance Standards for Antimicrobial Susceptibility Testing (μg/ml)
Sulfadiazine (SDZ)	1024	>=350
Sulfamethoxazole (SMZ)	512	>=350
Trimethoprim (TMP)	0.25	>=16
Sulfamethoxazole /Trimethoprim (SXT)	4	>=4

stimulate the production of ROS in *V. splendidus*. Meanwhile, the expression of *arcA* gene was also upregulated to 1.4- and 2.48-fold at 30 and 45 min, respectively (Fig. 3e), compared with that in the cells without contact of Cu^{2+}.

ROS affected the expression of *arcA* gene

To see if ROS could regulate the expression of *arcA* gene, a ROS inhibitor, N-Acetyl-L-cysteine (NAC), was applied. The effect of NAC on growth of *V. splendidus* strain Vs was first determined. 0.01 mM, 0.1 mM, and 1 mM NAC exhibited approximately the same trend without affecting growth (Fig. 4a). Then, the concentrations of NAC used to determine its affection on ROS production were 0.01, 0.1, and 1 mM. Under the three tested levels, addition of NAC inhibited ROS production in *V. splendidus* strain Vs. The quantity of ROS decreased to 88.3%, 81.4%, and 53.1% at 15, 30, and 45 min, respectively, after the addition of NAC (Fig. 4b). One millimole NAC showed approximately 50% inhibitory effect on ROS production without affecting growth (Fig. 5), so 1 mM NAC was added into bacterial culture to test its effect on the expression of *arcA* gene. The expression of *arcA* gene

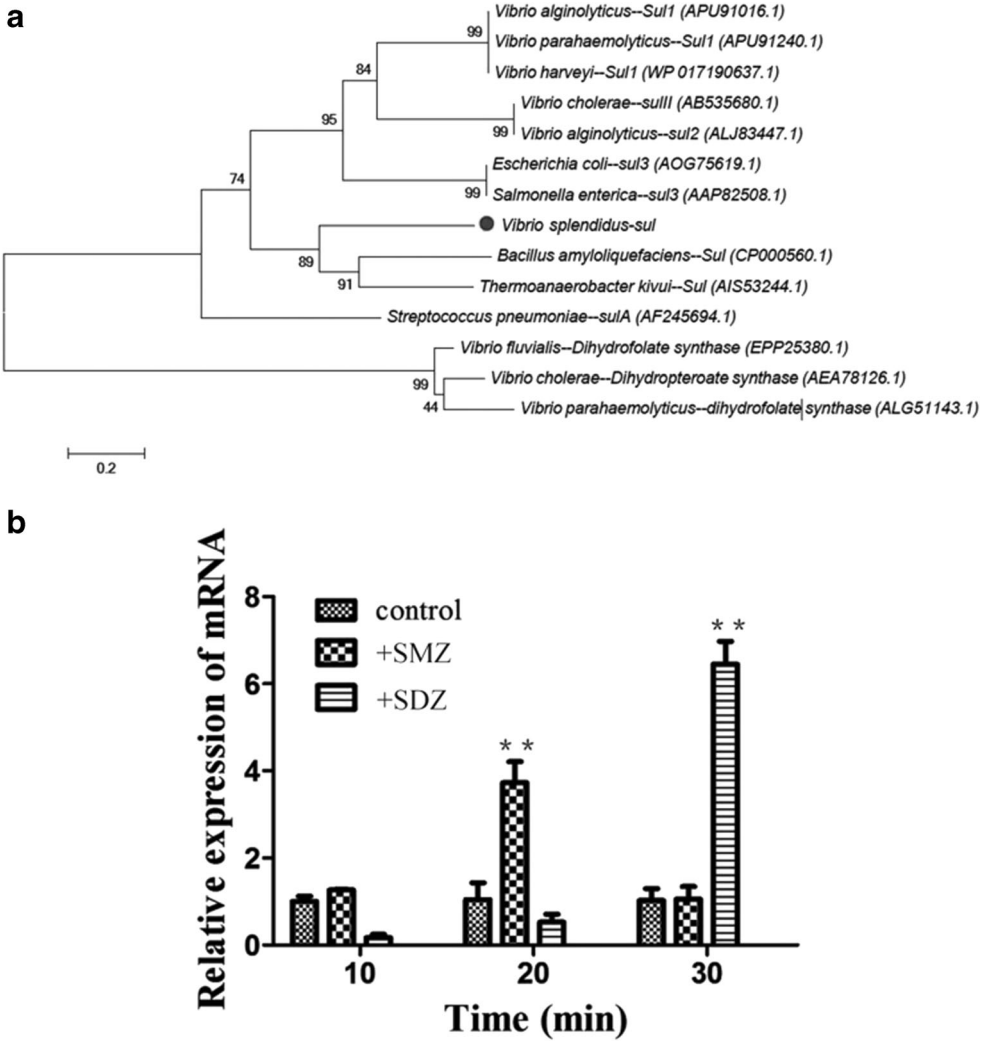

Fig. 1 Phylogenetic tree of *suls* from *V. splendidus* strain Vs and other bacteria constructed using MEGA 5.1 and CLUSTAL X (**a**). The one signed with dot indicated sul_{Vs} from *V. splendidus* strain Vs. Fold differences in mRNA level of sul_{Vs} in the presence of SDZ or SMZ (**b**). mRNA level of sul_{Vs} was normalized to that of 16S rRNA at each time point, and the mRNA level of sul_{Vs} in the cells treated with SDZ or SMZ was compared to that in the control cells at each time point. Data are the means for three independent experiments and are presented as the means ± SD. **, $P < 0.01$

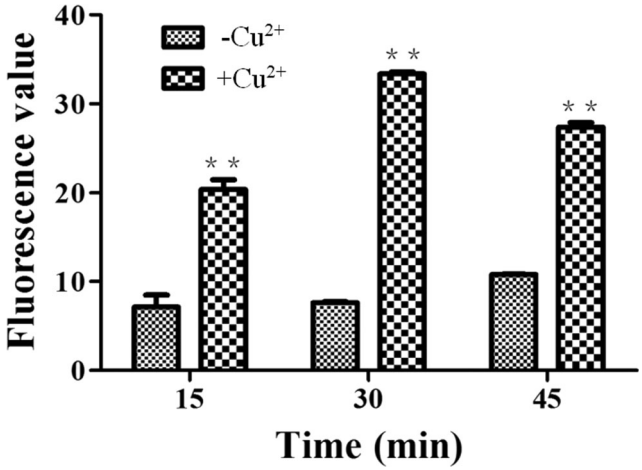

Fig. 2 Fluorescence value of ROS in the presence of Cu^{2+}. Samples were treated using DCFH-DA probe and the intracellular ROS production was qualified. The fluorescence value in Cu^{2+}-treated cells was compared to that in the control cells at each time point. Data are the means for three independent experiments and are presented as the means ± SD. **, $P < 0.01$

decreased to 26% in the presence of 1 mM NAC compared to that without NAC treatment (Fig. 6). So, it suggested that ROS could regulate the expression of *arcA* gene.

Cu^{2+} regulated the expression of *arcA* through ROS pathway

Since Cu^{2+} could simultaneously upregulate ROS and expression of *arcA,* we wondered whether Cu^{2+} regulated *arcA* expression through ROS or not. This was realized through the addition of NAC. When the NAC and Cu^{2+} were simultaneously added into the culture, ROS production decreased to 77.7%, 78%, and 50.6% at 15, 30, and 45 min, respectively, while ROS production was upregulated to 4.37-fold at 30 min when only Cu^{2+} was added (Fig. 5). Similarly, after NAC and Cu^{2+} were simultaneously added into culture for 30 or 45 min, the expression of *arcA* also decreased to 67% or 80%, respectively, while expression of *arcA* was upregulated to 1.4- and 2.3-fold in the presence of Cu^{2+} alone at these two time points (Fig. 6).

ArcA directly regulated expression of sul_{vs} gene

Prediction of the 1000 bp upstream of sul_{vs} gene showed that one promoter, named $Psul_{Vs}$, showed an ArcA binding site (Fig. 7a). $Psul_{Vs}$ was cloned into promoterless plasmid pSC11 and it developed blue colonies on X-gal agar plates, which suggested that $Psul_{Vs}$ was an active promoters. To further detect whether ArcA could directly bind to the promoter region of $Psul_{Vs,}$ recombinant ArcA was firstly obtained. *orf* of *arcA* is 717 bp, and it codes a protein of 238 aa with an estimated molecular mass of 27.1 kDa, and a theoretical pI of 5.29. Comparison with the known ArcA amino acid sequence indicated that the best matches for the amino acid sequence of ArcA were those of *Vibrio tasmaniensis* (CAV17486.1) and *Vibrio alginolyticus* (WP_115385736.1), which showed, respectively, 100% and 99% identity to the sequence ArcA in *V. splendidus* strain Vs. Pure recombinant

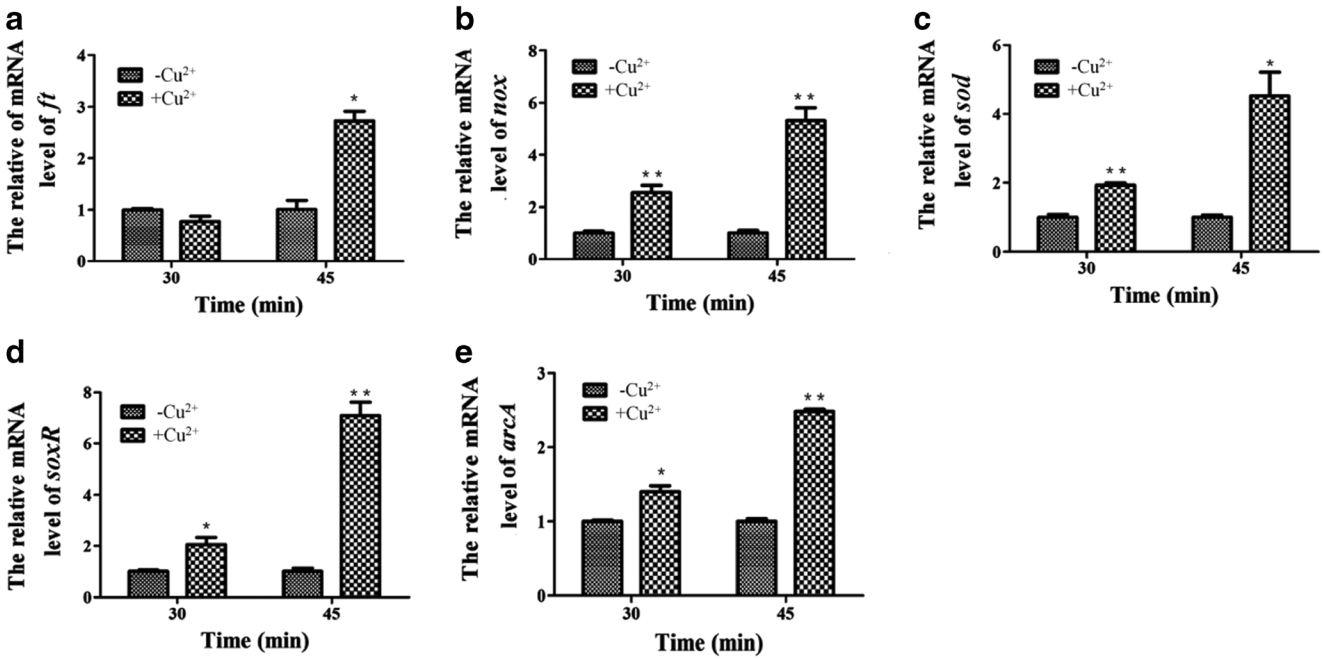

Fig. 3 Fold difference in mRNA levels of antioxidant-related genes in the presence of Cu^{2+} (**a** is for *ft*, **b** is for *nox*, **c** is for *sod*, **d** is for *soxR*, and **e** is for *arcA*). The mRNA level of each gene was normalized to that of 16S rRNA, and the mRNA level of each gene in the Cu^{2+}-treated cells was compared to that in the control cells at each time point. Data are the means for three independent experiments and are presented as the means ± SD. *, $P < 0.05$ and **, $P < 0.01$

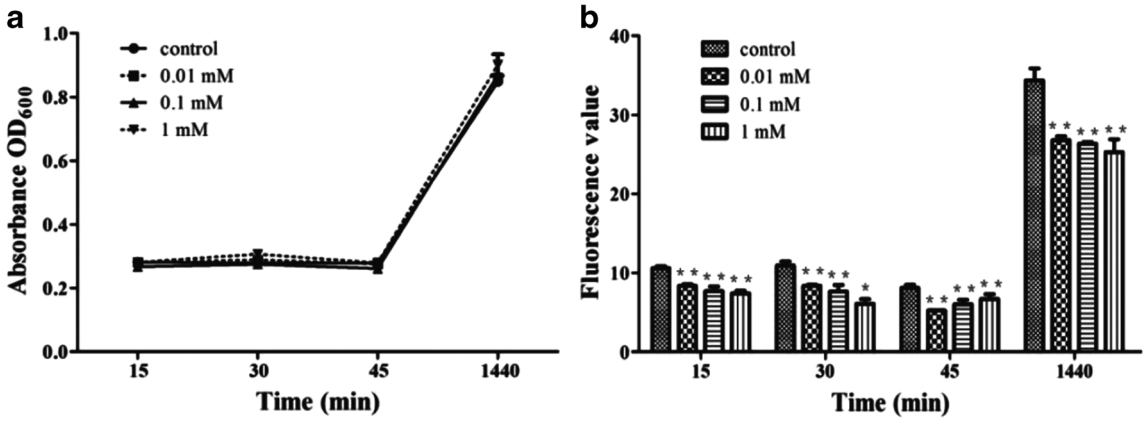

Fig. 4 OD_{600} (**a**) and ROS production (**b**) in the presence of different concentration of NAC. 0.01 mM, 0.1 mM, or 1 mM NAC were respectively supplemented into the culture medium. After cultured for 15, 30, 45 min, and 24 h, aliquots were taken for the measurement of absorbance at 600 nm (**a**), and remaining samples were used for ROS detection (**b**). The fluorescence value in NAC-treated cells was compared to that in the control cells at each time point. Data are the means for three independent experiments and are presented as the means ± SD. *, $P < 0.05$, and **, $P < 0.01$

ArcA was purified from BL21(DE3)/pET28a-*arcA* induced by IPTG under native condition.

Purified active recombinant ArcA, $Psul_{Vs}$ DNA, and negative control DNA were obtained for EMSA. A distinctive protein band for the purified ArcA was detected (Fig. 7b). The EMSA result indicated that ArcA could bind to $Psul_{Vs}$ with specificity, which predicted to possess the potential ArcA binding site, while it could not bind to the other DNA fragment (Fig. 7c, d). It indicated that ArcA was a transcriptional factor of sul_{Vs}. Taken together, these results demonstrated that ArcA could regulate the expression of sul_{Vs} gene, probably via direct interaction with the promoter region of sul_{Vs}.

Discussion

Although strains of *Vibrio* sp. had been reported to show resistance to different antibiotics (Okoh and Igbinosa 2010), our study was the first to report *V. splendidus* showed sulfonamide resistance to SDZ, SMZ, and SXT. The MIC of SDZ was 2.93-fold higher than the Performance Standards for Antimicrobial Susceptibility Testing (Jorgensen and Ferraro 2009). sul_{Vs} was homology to *sul3*, a sulfonamide resistance gene occurring in *Salmonella* sp. carrying class 1 integron with *aadA* and *dfrA* gene cassettes (Antunes et al. 2005). High antibiotic resistances have been frequently associated with bacterial pathogenesis (Beceiro et al. 2013); thus, the multiple antibiotic resistances may facilitate the infective ability of *V. splendidus* strain Vs. In natural environment, antibiotic resistance and heavy metals resistance are very frequently associated in the same organism, and furthermore, both resistances were due to the genes in the same plasmid,

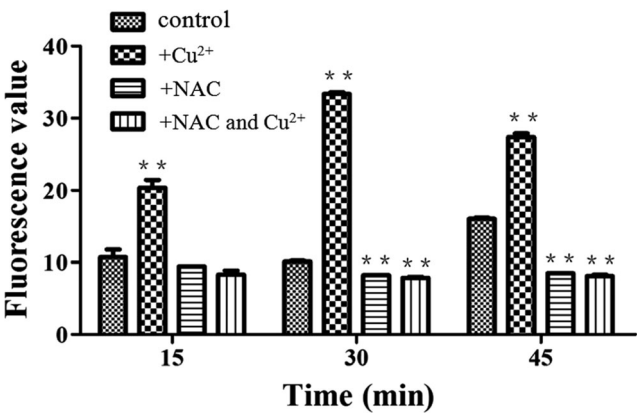

Fig. 5 Fluorescence value of ROS in the presence of Cu^{2+}, NAC or both Cu^{2+} and NAC. The fluorescence value in cells treated with Cu^{2+}, NAC or both Cu^{2+} and NAC was compared to that in the control cells at each time point. Data are the means for three independent experiments and are presented as the means ± SD. **, $P < 0.01$

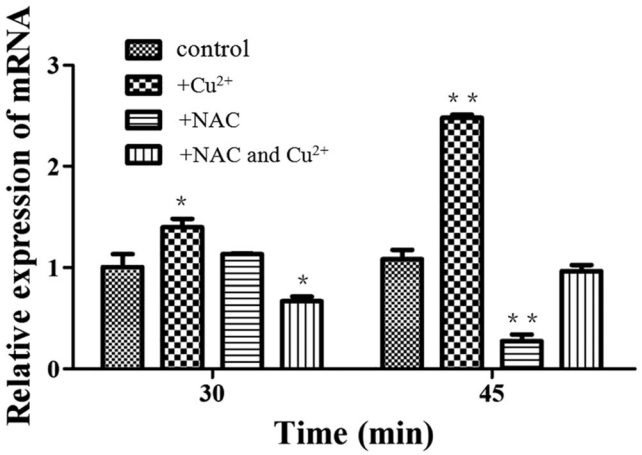

Fig. 6 Fold difference in mRNA level of *arcA* gene in the presence of Cu^{2+}, NAC or both Cu^{2+} and NAC. The mRNA level of *arcA* gene was normalized to that of 16S rRNA, and the mRNA level of *arcA* gene in cells treated with Cu^{2+}, NAC or both Cu^{2+} and NAC was compared to that in the control cells at each time point. Data are the means for three independent experiments and are presented as the means ± SD. *, $P < 0.05$ and **, $P < 0.01$

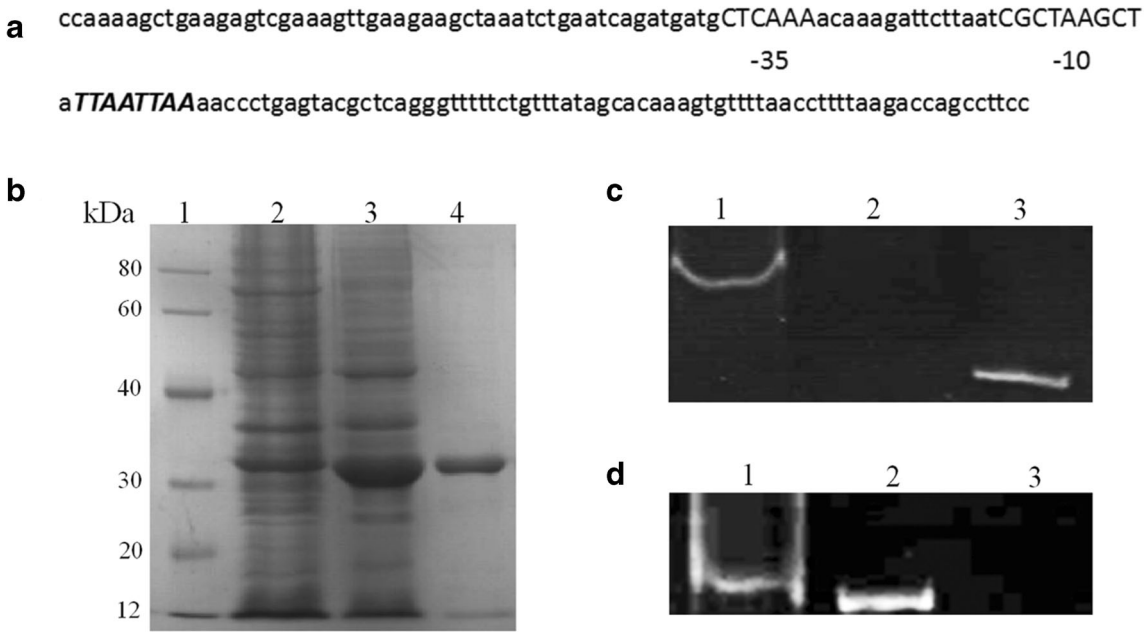

Fig. 7 The prediction of promoter regions of sul_{Vs} and binding sites of ArcA (**a**). The − 35 and − 10 regions of the promoter $Psul_{Vs}$ have been identified. Bold and italic part represents the ArcA binding site, predicted using both BPROM and consistent sequence of 5′- nGTTAATTAn-3′(n is A or T) in *E. coli* (Lynch and Lin 1996). Recombinant ArcA was purified from BL21(DE3)/pET28a-arcA (**b**). Lane 1, protein Marker; lane 2, proteins from un-induced BL21(DE3)/pET28a-arcA; lane 3, proteins from induced BL21(DE3)/pET28a-arcA; lane 4, purified ArcA. Specific binding of ArcA to the promoter regions of sul_{Vs} determined by EMSA (**c**). Lane 1, ArcA and $Psul_{Vs}$; lane 2, ArcA; lane 3, $Psul_{Vs}$. A 303 bp DNA fragment from the upstream of promoter was used as a negative control (**d**). Lane 1, ArcA and negative DNA control; lane 2, negative DNA; lane 3, ArcA

transposon, or integrin (Baker-Austin et al. 2006; Graham et al. 2011). Cu^{2+} has been reported to be closely related to ARGs (Berg et al. 2005). Cu^{2+} treatment has positive correlation to the erythromycin resistance genes in urban wastewater (Gao et al. 2015), carbapenem antibiotic (Caille et al. 2007), and tetracycline, erythromycin, and β-lactams (Graham et al. 2011). Our study was the first time to report that Cu^{2+} could significantly upregulate the expression of sul_{Vs}.

Although Cu^{2+} is an essential microelement that is necessary for a wide range of metabolic processes in living organisms, an accumulation of an over-dosage of Cu^{2+} can be toxic to bacteria with strain specificity (Ali et al. 2013). Similar to the effects of other heavy metals, excess of Cu^{2+} was also associated with ROS production (Luna et al. 2015). In our present study, Cu^{2+} stimulated the ROS production in *V. splendidus*, similar to that occurred in *E. coli* (Santo et al. 2011a) and *Candida albicans* (Santo et al. 2011b). Furthermore, we tested the response of several antioxidant enzymes, including SoxR, the redox regulator of the expression of SOD (Imlay 2008), NADH oxidase (Nox), one enzyme produce H_2O_2 which activates SOD and removes ROS (Gui et al. 2008), and Ferritin (Ft) that could alleviate the cytotoxicity caused by H_2O_2 and Fe^{2+} stresses, thus preventing the damage of [•OH] caused by Fenton reaction (Zhao et al. 2002; Imlay 2008). The upregulation of these antioxidant enzyme genes further suggested that Cu^{2+} could stimulate the generation of ROS and the bacterial cells have to initiate the antioxidant processes to balance ROS level.

ArcA is a transcriptional regulator that plays an important role in tolerating ROS (Salmon et al. 2003). We found that NAC could inhibit the production of ROS in *V. splendidus*, just like what happened in the other bacterial such as *Listeria monocytegenes* (Zhang et al. 2013a), and *arcA* gene decreased responding to ROS level like in *E. coli* (Salmon et al. 2003; Loui et al. 2009). In *V. splendidus* strain Vs, after NAC was added, ROS was inhibited, followed by reduced expression of *arcA*, which indicated that the expression of *arcA* was related to ROS. Moverover, NAC could inhibit the upregulatory effects of Cu^{2+}, which indicated that Cu^{2+} could also regulate *arcA* through the ROS mediated pathway. And after that, ArcA acted as a transcriptional regulator of sul_{Vs} by directly and specifically binding to *sul* promoter region.

Taking all the results and references together, we proposed the following regulation pathway of Cu^{2+} on sul_{Vs} expression in *V. splendidus* strain Vs: Cu^{2+} stimulated ROS production and higher ROS upregulated the expression of *arcA* gene. ArcA directly bound to the promoter region of sul_{Vs} and upregulated the expression of sul_{Vs} gene. Our study provides a basis for studying the combination effects of environmental factors on bacterial resistance.

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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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