Proteomics Analysis Reveals Bacterial Antibiotics Resistance Mechanism Mediated by ahSlyA Against Enoxacin in Aeromonas hydrophila

Zhen Li1,2,3,4, Lishan Zhang1,3, Qingli Song1,3, Guibin Wang1,5, Wenxiao Yang1,3, Huamei Tang1,3, Ramanathan Srinivasan1,3, Ling Lin1,3 and Xiangmin Lin1,3,4*

OPEN ACCESS

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*Correspondence:
Xiangmin Lin
xiangmin@fafu.edu.cn

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 23 April 2021
Accepted: 17 May 2021
Published: 08 June 2021

Bacterial antibiotic resistance is a serious global problem; the underlying regulatory mechanisms are largely elusive. The earlier reports state the vital role of transcriptional regulators (TRs) in bacterial antibiotic resistance. Therefore, we have investigated the role of TRs on enoxacin (ENX) resistance in Aeromonas hydrophila in this study. A label-free quantitative proteomics method was utilized to compare the protein profiles of the ahSlyA knockout and wild-type A. hydrophila strains under ENX stress. Bioinformatics analysis showed that the deletion of ahSlyA triggers the upregulated expression of some vital antibiotic resistance proteins in A. hydrophila upon ENX stress and thereby reduce the pressure by preventing the activation of SOS repair system. Moreover, ahSlyA directly or indirectly induced at least 11 TRs, which indicates a complicated regulatory network under ENX stress. We also deleted six selected genes in A. hydrophila that altered in proteomics data in order to evaluate their roles in ENX stress. Our results showed that genes such as AHA_0655, narQ, AHA_3721, AHA_2114, and AHA_1239 are regulated by ahSlyA and may be involved in ENX resistance. Overall, our data demonstrated the important role of ahSlyA in ENX resistance and provided novel insights into the effects of transcriptional regulation on antibiotic resistance in bacteria.

Keywords: antibiotics resistance, AhSlyA, enoxacin, quantitative proteomics, Aeromonas hydrophila

INTRODUCTION

Antibiotic-resistant bacterial strains were discovered over 90 years ago. Since then, antibiotic-resistant bacterial strains have been found to be widely distributed in various environments, such as in hospitals, seafood, and aquaculture farms, and as a result, they pose a serious public health problem worldwide (Fritsche et al., 2009; Peng et al., 2015). Although, several mechanisms of antibiotic resistance such as membrane permeability, plasmid transfer, antibiotic modification or
degradation, efflux, and biofilm formation have been described in recent years, the underlying mechanism of acquire antibiotic resistance in bacteria is still largely unknown (Peterson and Kaur, 2018). The emergences of drugs resistant bacterial strains have been caused by many complicated characteristics, one of which is the antimicrobial resistance genes (ARGs) transcriptional regulators (TRs). Bacterial TRs play an important role in the transcriptional regulation of functional genes needed to survive environmental stresses, including antibiotic resistance (Zhou and Yang, 2006; Tang et al., 2019a). For example, a mutation in the multiple antibiotic resistance (MarR) TRs in Escherichia coli has been shown to lead to the expression of the marRAB operon. Therefore, it promotes the expression of the transcription factor MarA and activation of the acrAB and tolC efflux pump genes, resulting in multi-drug resistance to tetracycline, quinolones, β-lactams, and phenolic compounds (Pourahmad Jaktaji and Ebadi, 2013; Lankester et al., 2019). Additionally, the TR EmrR is an inhibitor of efflux pump EmrCAB in Chromobacterium violaceum and the mutation of emrR92H increases the resistance of C. violaceum to nalidixic acid (Barroso et al., 2018). Nonetheless, there are hundreds of bacterial TRs with biological functions that are poorly characterized and most of the direct and indirect effects of TRs on bacterial antibiotic resistance are largely unknown.

Aeromonas hydrophila is a widely distributed environmental bacterium and a well-known fish pathogen. The use of antibiotics in aquaculture industries has resulted in the emergence of multi-drug resistant A. hydrophila strains in aquaculture and even in hospital settings (Li et al., 2020; Zhu et al., 2020). In recent years, many research, including our previous study, have been found that the several metabolic pathways related genes were involved in the drug-resistance on this pathogen byomics technologies (Hossain et al., 2018; Sun et al., 2019; Li et al., 2021). In our previous study, we reported that the LysR-type TR YeeY in A. hydrophila plays an important role in the regulation of furazolidone resistance by directly regulating ARGs, including AHA_3222 and AHA_4275. It indicates the crucial role of TRs in the antibiotic resistance of this pathogen. However, the underlying mechanisms of antibiotic resistance regulated by TRs in A. hydrophila are needed to be further investigated.

To better understanding the role and regulatory mechanism of TR on the bacterial physiological function. In this study, we reported on a MarR family TR in A. hydrophila, AhSlyA (gene name ahSlyA or AHA_1240). AhSlyA is a winged helix-turn-helix (wHTH) DNA-binding TR. Previous research reported the homologous proteins of this TR in other bacterial species play diverse biological functions such as cell metabolism and virulence, while its biological effect and molecular mechanism are still largely unknown, especially for the bacterial antibiotics resistance in A. hydrophila (Banda et al., 2019; Tian et al., 2021). In this study, we constructed an ahSlyA deletion mutant (ΔahSlyA) in A. hydrophila and found that it displayed significantly decreased resistance to the quinolone antibiotic, enoxacin (ENX), as compared to the wild-type (WT) parent strain. To further investigate the direct or indirect effect of this TR on the antibiotic resistance of A. hydrophila, a label-free quantitative proteomics method was used to compare the differentially expressed proteins between the ΔahSlyA and WT strains under ENX stress. Moreover, a several differentially expressed genes were deleted and their antibiotic susceptibility to ENX was validated. This study will conduce to further understand the complicated antibiotic resistance mechanisms mediated by bacterial TRs.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

The strains used in this study are A. hydrophila ATCC 7966, ΔahslyA, and the ahSlyA complemented strain. E. coli MC1061 and S17 were stored in our laboratory previously. The culture temperature of A. hydrophila and E. coli were 30 and 37°C, respectively. Both bacterial strains were cultured in Luria–Bertani (LB) medium with appropriate antibiotics.

**Construction of the Gene Deletion Strain**

The deletion strain was constructed based on the principle of two-step homologous recombination using the suicide vector pRE112, as previously described (Yu et al., 2005). Briefly, the pRE112 plasmid fused with about 500-bp of the upstream and downstream flanking regions of the target gene was constructed using A. hydrophila ATCC 7966 genomic DNA as template and then transferred into competent E. coli MC1061 cells. Then, the plasmid of a positive clone was extracted and transferred into E. coli S17 competent cells. After verifying by PCR amplification, the E. coli S17 carrying the pRE112 recombinant vector was then conjugated with A. hydrophila in a 4:1 ratio to the first step of homologous recombination. Positive A. hydrophila clones were selected on LB agar plates with ampicillin and chloramphenicol (Yeasen Inc., Shanghai, China). The second step of homologous recombination was carried out in LB medium containing 20% sucrose. The ΔahslyA in A. hydrophila was confirmed by plating chloramphenicol, followed by PCR and DNA sequencing. Finally, after about 20 generations of stable inheritance and correct DNA sequencing, the ΔahslyA mutant was stocked and stored in the freezer at −80°C.

**Protein Sample Preparation**

Bacterial strains were inoculated in 5 mL LB medium, cultured for 16 h and then transferred at the ratio of 1:100 into 100 mL of LB medium containing ENX at a final concentration of 0.0078 µg/mL. After culturing for about 3 h (until the culture reached an OD_{600} of approximately 1.0), the cells were collected by centrifugation at 8,000 × g at 4°C for 20 min and washed twice with PBS. The bacterial samples were then resuspended in 5 mL of PBS buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then lysed by ultra-sonication. The supernatant was collected by centrifugation at 8,000 × g for 20 min and the total protein concentration was detected via the Bradford method. About 50 µg of each protein sample was reduced with 50 mM dithiothreitol at 56°C, alkylated with 25 mM iodoacetamide in dark and then digested to peptides with a 1:20 ratio of trypsin (Promega Inc., Shanghai, China). The enzymatic peptides were desalted with a C18 column (Waters Inc., Milford, MA,
United States) and dried with a centrifrap concentrator (Labconco Inc., Kansas City, MO, United States). Each group sample was performed three independent repeats for biological replicates.

**Label-Free LC-MS/MS**
The desalted peptides were dissolved in liquid chromatography mobile phase buffer A [containing 2% acetonitrile, 0.1% formic acid (FA)], loaded onto the pre-column at a flow rate of 4.5 µL/min on the chromatographic system and then injected into the column at a flow rate of 300 nL/min by an easy-nlcl200 system (Thermo Scientific Inc., Waltham, MA, United States). The liquid gradient setting was as follows: 0–3 min, buffer B (containing 80% acetonitrile, 0.1% FA) increased linearly from 2 to 5%; 3–103 min, solution B increased linearly from 5 to 28%; 103–108 min, solution B increased linearly from 35 to 90%; 110–120 min, the solubility of solution B was maintained at 90%. Mass spectrometry was performed with an Orbitrap Fusion Lumos system (Thermo Scientific Inc., Waltham, MA, United States) nanospray ion source. The spray voltage was 2.0 KV and the ion transfer tube temperature was 300°C. We used the data-dependent acquisition mode to collect data. The parameters were as follows: the precursors from 350 to 1,600 m/z were scanned at a resolution of 60,000, and the AGC target was set at 4e5. For MS/MS, the HCD collision energy was 30% with a resolution of 15,000. The AGC target was set to 5e4. The cycle time was 3 s. All raw data were searched by Maxquant software v.1.6.3.4 against Uniprot A. hydrophila ATCC7966 database. Proteins with the number of peptides greater than 2, p-value less than 0.05, and protein ratio difference greater than 1.5 times were selected as differential proteins for bioinformatics analysis. The raw MS files were submitted to the iProx (Integrated Proteome resources) database under the accession number IPX0002908000 (Ma et al., 2019).

**Bioinformatics Analysis**
The GO (gene ontology) analysis of altered proteins were performed using the online software DAVID1 and visualized with GOplot package in R language software (Walter et al., 2015; Zhang L.S. et al., 2020). The protein-protein interaction (PPI) network was predicted using the String2 online database with a confidence score ≥ 0.7 and the network was clustered using the “Markov Cluster Algorithm (MCL)” and the inflation parameter was set as 4 (Szklarczyk et al., 2017). Finally, the visualized network diagram of PPI was drawn using Cytoscape 3.8.03 (Shannon et al., 2003).

**Determination of Minimal Bactericidal Concentrations**
The minimal bactericidal concentration (MBC) assay was performed by the agar dilution method, as previously described (Jiang et al., 2020). Briefly, an overnight bacterial culture was passaged into fresh LB medium, incubated at 30°C with shaking until the OD_{600} reached about 1.0 and then diluted 100 times. Then, 2 µL of each dilution was spotted onto an LB agar plates with twofold dilution gradient concentration of antibiotics (ciprofloxacin, levofloxacin, enoxacin, and enrofloxacin purchased from Yeasen biotech, Ltd., Shanghai, China), respectively, and incubated at 30°C for 16 h. Each experiment was performed at least three times with biological duplicates.

**RESULTS**

**ahslyA Mutant Affects Antibiotic Susceptibility in Aeromonas hydrophila**
In order to better understand the characteristics of the TR AhslyA on bacterial antibiotic resistance, we first constructed AhslyA mutant strain and then tested its antibiotic susceptibilities against various antibiotics, including several quinolone antibiotics. As shown in Figure 1, loss of ahslyA caused a twofold increase in the MBC to ciprofloxacin (CIP) and levofloxacin (LVX), and increased the MBC of ENX by four times; however, it did not affect the susceptibility to enrofloxacin (ENR). Moreover, complementation of the AhslyA strain restored the antibiotic susceptibilities similar level to the WT strain, which is suggesting that the ahslyA gene in A. hydrophila is involved in the regulation of several antibiotic resistances, especially ENX.

**Quantitative Proteomics Comparison Between WT and AhslyA Strains Under ENX Stress**
In order to further investigate the regulatory mechanism of ahslyA on ENX antibiotic resistance, we isolated whole protein samples from WT and AhslyA strains with or without exposure to 0.0078 µg/mL ENX treatments. After trypsin digestion, each sample was quantified by a label-free quantitative proteomics method to compare the differentially expressed proteins between both groups. As shown in Figure 2A, positive correlations greater than 0.98 were found between the intensities of the MS of each sample with the regression coefficients, which indicated that the quantitative analysis results of MS in this study were stable and reliable. A principal component analysis (PCA) scatter diagram was drawn to cluster the samples. The dots of different colors in Figure 2B represent three groups of repeats of the same sample. It can be seen from the diagram that the three dots of the same color are relatively close; indicating that the gene expression pattern of the three repeats of the same sample had a small difference and suitable data repeatability. The proteomic analysis of the AhslyA + ENX group was significantly separated from the WT + ENX group in the direction of PC1, indicating that there may be significant differences between them. These results indicate that the effect of the deletion of ahslyA on bacterial proteome is more than the effect of ENX stress.

**The Differential Protein Abundances of WT and AhslyA in Response to ENX Stress**
In this study, a total of 2,534 proteins were identified by mass spectrometry (unique peptide number ≥ 2 and false
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FIGURE 1 | Measurement of the MBCs of ahSLyA derivatives in A. hydrophila to four quinolone antibiotics. (A) The MBCs of ΔahSLyA and complemented ΔahSLyA strains, in addition to WT and ΔahSLyA carrying an empty vector as negative controls, were tested for their sensitivity to (A) ciprofloxacin, (B) levofloxacin, (C) enoxacin, and (D) enrofloxacin. The MBCs of each strain to antibiotics were compared with WT as MBC fold change.

discovery rate (FDR) < 1%). The protein abundance ratio that was ≥ 1.5 (upregulated expression) and ≤0.667 (downregulated expression) with a p-value < 0.05 of each compared group was regarded as differentially expressed proteins. We analyzed the data by two comparisons, ΔahSLyA + ENX vs. ΔahSLyA and ΔahSLyA + ENX vs. WT + ENX, in order to interpret the ahSLyA mediated ENX resistance in this study. When compared with ΔahSLyA without antibiotic treatment, there was 49 differentially expressed proteins were found, which accounted for 1.77% of the total identified proteins, including 35 increasing and 14 decreasing in abundance in the ΔahSLyA + ENX treatment group (Figure 2C). When compared with WT + ENX, a total of 172 proteins, including 101 increasing and 71 decreasing in abundance, were altered in the ΔahSLyA + ENX treatment group (Figure 2D). The following overlap analysis between both group comparisons showed that ΔahSLyA + ENX vs. ΔahSLyA and ΔahSLyA + ENX vs. WT + ENX have 14 common altered proteins, of which seven have the same protein expression trend and another seven have the opposite expression (Supplementary Table 1 and Figure 2E).

Bioinformatics Analysis of Altered Proteins in ΔahSLyA + ENX/ΔahSLyA and ΔahSLyA + ENX/WT + ENX Comparisons

We then used GO terms enrichment to analyze altered proteins in both comparisons. In the biological process (BP) enrichment, the DNA metabolic process, cellular response to stimulus, SOS response, and cell communication were the most enriched terms in the ΔahSLyA + ENX vs. ΔahSLyA comparison. Additionally, the response to stress, the response to stimulus, the DNA metabolic process, and the cellular response to external stimulus were the most enriched terms in the ΔahSLyA + ENX vs. WT + ENX comparison. When comparing both group, the response to stress and the cellular response to stress in the ΔahSLyA + ENX vs. WT + ENX comparison changed significantly, while no change were observed in the ΔahSLyA + ENX vs. ΔahSLyA comparison (Figures 3A,B).

We further analyzed the predicted PPI network of altered proteins in both comparisons using STRING software and then clustered them using the MCL algorithm. In the ΔahSLyA + ENX vs. ΔahSLyA comparison, there were five clusters that were enriched (Figure 4). Eight increasing proteins were clustered in cluster 1, and most of them were SOS or DNA repair-related proteins. There were six metabolic pathway-related proteins clustered in cluster 2, including three proteins that might be involved in acyl-CoA metabolism, namely, acyl-CoA thioester hydrolase YciA (gene name AHA_1563), acyl-CoA thioesterase I (AHA_3489), and acyl carrier protein (acpP). Both AHA_3297 and AHA_0044 are a sensor histidine kinase and sensory box/GGDEF family gene, respectively, which were present in clusters 3–5. It indicates that ahSLyA may affect a bacterial two-component or cyclic di-GMP signaling system. The top 14 clusters of the ΔahSLyA + ENX vs. WT + ENX
comparison are shown in Figure 3B. In cluster 1, at least seven proteins, including RecA, UvrA, UvrD, LexA, DinB, RecN, and RecG are involved in DNA repair processes and all seven were decreasing in abundance. AHA_3525 is a response regulator, which was the network hub in cluster 2. Moreover, AHA_3525 interacted with three TRs, namely, AHA_0137 (response
regulator, GltR), AHA_1862 (response regulator protein), and AHA_3966 (DNA-binding response regulator), in addition to AHA_3297 (diguanylate cyclase DosC), and most of these genes were increasing in abundance. In cluster 3, most of altered proteins were metal ion-related proteins. For example, SelA, SelD, and the SelT/SelW/SelH domain (AHA_1610) were involved in selenium metabolism in prokaryotes. Additionally, clusters 4–6 were mostly clustered in oxidative respiration, uncharacterized protein and sulfate metabolism.

The ENX-Resistance Capabilities of Proteins Regulated by ahslyA
In order to better understand the ENX resistance mechanism mediated by the TR ahslyA, we further assessed the effect of several gene mutants on the resistance to ENX, which were shown to be regulated directly or indirectly by ahslyA in our proteomics results. Selected gene deletion strains, including three genes related to decreasing in protein abundance (AHA_0655, AHA_1195, and AHA_3721) and three genes related to increasing in protein abundance (AHA_1239, AHA_2114, and narQ) in the ΔahslyA + ENX vs. WT + ENX proteomic comparison, were successfully constructed via a two-step homologous recombination method using the primer pairs listed in Supplementary Table 2. Each mutant was assessed for sensitivity to ENX using an antibiotic susceptibility assay. As showed in Figure 5, the ΔAHA_0655 exhibited a slightly decreased resistance while other mutants showed no significant difference to 0.0078 µg/mL of ENX. The ΔAHA_2114 and ΔnarQ showed a slight decrease in resistance to 0.01 µg/mL of ENX, and the ΔAHA_1239 and ΔAHA_3721 demonstrated significantly increased resistance to 0.01 µg/mL of ENX. These results suggested that ahslyA may regulate the transcription of AHA_0655, narQ, AHA_3721, AHA_2114, or AHA_1239 to against ENX stress.

DISCUSSION
It is well known that bacterial TRs play a crucial regulatory role in diverse physiological and pathological functions. However, as there are hundreds of TRs are identified in prokaryotes and only a few of them have been well described, the intrinsic regulatory mechanisms of prokaryotic TRs are largely unknown (Brown et al., 2003). SlyA belongs to the TR MarR family that possesses a winged-helix DNA binding domain (Wilkinson and Grove, 2006). It was first reported to regulate virulence in many pathogens, such as Salmonella typhimurium, Enterococcus faecalis, and Dickeya dadantii (Libby et al., 1994; Haque et al., 2009; Michaux et al., 2011b). It has also been shown to play important roles in oxidative stress, bile salt stress, antimicrobial peptide resistance, heat, and acid stress (Buchmeier et al., 1997; Spory et al., 2002; Shi et al., 2004; Michaux et al., 2011a). Therefore, it suggests that this TR plays an important role in numerous physiological functions. However, the mechanism underlying these biological functions remains elusive.

Since SlyA plays an important role in the resistance to environmental stresses, we speculated that it may contribute to bacterial antibiotic resistance, because antibiotics are toxic small molecules like bile salts. To test our hypothesis, the antibiotic susceptibilities of the slyA gene deletion mutant and its complemented strains were analyzed in this study. We found that the ΔahslyA had increased resistance against several quinolone antibiotics, suggesting that this TR may negatively regulate resistance to certain antibiotics, especially ENX. We then performed label-free quantitative MS to characterize the effect of ahslyA gene deletion on the proteome of A. hydrophila cells with or without ENX treatment. Here, we analyzed two group comparisons in order to understand the ENX resistance mediated by ahslyA. In the ΔahslyA + ENX vs. ΔahslyA, we identified 49 altered proteins, and when ΔahslyA + ENX was compared to WT + ENX, there were 172 differentially expressed proteins. GO bioinformatics analysis showed that both comparisons were related to a stress response or stimulus, which indicated that ahslyA may play an important role in stress resistance to environmental factors, including antibiotics. Although, we have not obtained a significant KEGG (Kyoto Encyclopedia of genes and genes) pathway enriched altered proteins in either of the comparisons in this study. The PPI prediction plus the MCL
algorithm showed that these altered proteins can be classified into several clusters. In the ΔahslyA + ENX vs. ΔahslyA comparison, five DNA repair-related proteins (RecA, RecN, RuvA, UvrA, and UrvD) were significantly increased in abundance. It is well known that quinolone antibiotics inhibit DNA synthesis and cause DNA strand cleavage or cell death. The underlying mechanism of quinolone antibiotic resistance in bacteria is due to the upregulation of DNA repair related proteins. In our previous study, 11 SOS responses or DNA repair-related proteins of A. hydrophila were reported to be increased under ENX stress (Zhang L.S. et al., 2020). Moreover, the deletion of uvrA decreased the ENX tolerance in A. hydrophila, which suggests the important role of the DNA repair process in protecting the DNA from quinolone-induced damage. Interestingly, we found that many of the DNA repair-related proteins decreased in abundance in the ΔahslyA + ENX vs. WT + ENX comparison. This could have happened for a few reasons. First, the loss of ahslyA likely slowed down the toxic effect of ENX, so that bacteria did not trigger the DNA repair system. Second, the DNA repair response should be a last resort against ENX, as it may cause genetic mutations that influence bacterial survival. Third, ahslyA may regulate other drug resistance genes, aside from just DNA repair processes.

In both proteomic comparisons, the ΔahslyA + ENX vs. WT + ENX comparison may be better than the ΔahslyA + ENX vs. ΔahslyA comparison to interpret ahslyA-mediated ENX resistance, because the first comparison is on the same ENX background. Therefore, we focused more on the properties of altered proteins between ΔahslyA + ENX compared to ΔahslyA. Of these altered proteins, we found ahslyA could directly or indirectly regulate at least 11 TRs (gltR, yidZ, ycaN, citA, AHA_3297, AHA_0117, AHA_3721, AHA_1240, AHA_4233, AHA_1862, and AHA_3966), which indicating that these TRs may construct a complicated gene regulatory network to maintain the intracellular homeostasis during ENX stress. Of these TRs, AHA_3966 is homologous with E. coli ompR, with an identity of 49%. The ompR gene belongs to a well-known two-component regulatory system and plays important roles in multiple physiological functions, including antibiotic resistance (Lin et al., 2012; Zhang M.M. et al., 2020). Moreover, both AHA_1862 and AHA_3297 coded diguanylate cyclase and AHA_3525 coded phosphodiesterase that govern the cellular level of c-di-GMP, which acts as a unique bacterial second messenger to trigger various cellular responses, such as in motility, biofilm formation, and antibiotics resistance (De et al., 2008;
increased 3,899 folds in the ΔahslyA + ENX vs. WT + ENX comparison. Interestingly, AHA_1239 is the downstream gene of ahSlyA (AHA_1240), and its amino acid sequence has 30% identity to multi-drug resistance protein MdtN in E. coli. Moreover, the deletion of ahSlyA and exposure to ENX stress also caused an upregulation of A0KK37 (AHA_2114), which is the multi-drug resistance protein MdtK. Since both proteins are members of the MATE (multi-drug and toxic compound extrusion) protein family. Further, the overexpression of MdtK increases resistance to norfloxacin, doxorubicin, and acriflavine in Salmonella enterica serovar Typhimurium. More, the ahSlyA may negatively regulate both MdtN and MdtK to obtain ENX resistance in this study (Nishino et al., 2006). Additionally, the TRs A0KHI5 (AHA_1195, YcaN) and A0KPG8 (AHA_3721), nitrate sensor protein, A0KIL7 (narQ), belong to a two-component regulatory system play important roles on diverse biological functions in other bacterial species (Oshima et al., 2002). In this study, A0KHI5 and A0KPG8 were down-regulated, while NarQ increased in the ΔahslyA + ENX vs. WT + ENX proteomics data, suggesting that they may affect ENX resistance regulated by ahSlyA.

To test our hypothesis, the antibiotic susceptibilities of these six gene deletion mutants were determined. Our results showed that the deletion of AHA_0655 slightly decreased the growth of A. hydrophila under low dose of ENX stress. Further the deletion of AHA_2114 and narQ significantly decreased bacterial growth under high dose of ENX stress, while the deletion of AHA_3721 increased antibiotic susceptibility in A. hydrophila. Which suggested that ahSlyA may regulate AHA_0655, narQ, AHA_3721, and AHA_2114 for ENX resistance. Although AHA_1239 may act as a multi-drug resistance protein, the deletion of AHA_1239 caused significant resistance to ENX in this study. The inherent reason is unknown, but based on the fact that AHA_1239 is the downstream neighbor of ahSlyA, the deletion of AHA_1239 may affect the expression of ahSlyA and then trigger other ARGs or systems against ENX stress. Overall, our data demonstrated the important role of ahSlyA in the multiple ARGs regulation during ENX resistance and provided novel insights into the effects of TRs on the antibiotic resistance of bacteria.

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: http://www.proteomexchange.org/, PXD024843.

AUTHOR CONTRIBUTIONS

XL and LZ conceptualized and validated the study. ZL, LL, and LZ performed the methodology and the data curation and wrote the manuscript for the final draft. ZL, GW, and QS performed the formal analysis. XL was responsible for the resources, supervised
the study, and performed the funding acquisition. RS, WY, HT, and XL wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was sponsored by grants from Key projects of Natural Science Foundation of Fujian Province (No. 2020J02023), NSFC projects (Nos. 31670129 and 31802343), China Post-doctoral Science Foundation (Grant No. 2019M662214), and Program for Innovative Research Team in Fujian Agricultural and Forestry University (No. 712018009).

ACKNOWLEDGMENTS

We acknowledge the support of the program for Key Laboratory of Marine Biotechnology of Fujian Province (2020MB04) and the Fujian-Taiwan Joint Innovative Center for Germplasm Resources and Cultivation of Crop (FJ 2011 Program, No. 2015-75, China). We would also like to thank the help from Central Laboratory, Fujian Medical University Union Hospital, China.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.699415/full#supplementary-material

Supplementary Figure 1 | Construction and confirmation of the six gene deletion strains.

Supplementary Table 1 | The identification of 14 common altered proteins between both group comparisons using label-free analysis.

Supplementary Table 2 | The primer pairs used in this study.
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**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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