Characterization of the Role of Two-Component Systems in Antibiotic Resistance Formation in Salmonella enterica Serovar Enteritidis

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ABSTRACT The two-component system (TCS) is one of the primary pathways by which bacteria adapt to environmental stresses such as antibiotics. This study aimed to systematically explore the role of TCSs in the development of multidrug resistance (MDR) in Salmonella enterica serovar Enteritidis. Twenty-six in-frame deletion mutants of TCSs were generated from S. Enteritidis SJTUF12367 (the wild type [WT]). Antimicrobial susceptibility tests with these mutants revealed that 10 TCSs were involved in the development of antibiotic resistance in S. Enteritidis. In these 10 pairs of TCSs, functional defects in CpxAR, PhoPQ, and GlnGL in various S. Enteritidis isolates led to a frequent decrease in MIC values against at least three classes of clinically important antibiotics, including cephalosporins and quinolones, which indicated the importance of these TCSs to the formation of MDR. Interaction network analysis via STRING revealed that the genes cpxA, cpxR, phoP, and phoQ played important roles in the direct interaction with global regulatory genes and the relevant genes of efflux pumps and outer membrane porins. Quantitative reverse transcription-PCR analysis further demonstrated that the increased susceptibility to cephalosporins and quinolones in ΔphoP and ΔcpxR mutant cells was accompanied by increased expression of membrane porin genes (ompC, ompD, and ompF) and reduced expression of efflux pump genes (acrA, macB, and mdtK), as well as an adverse transcription of the global regulatory genes (ramA and crp). These results indicated that CpxAR and PhoPQ played an important role in the development of MDR in S. Enteritidis through regulation of cell membrane permeability and efflux pump activity.

IMPORTANCE S. Enteritidis is a predominant Salmonella serotype that causes human salmonellosis and frequently exhibits high-level resistance to commonly used antibiotics, including cephalosporins and quinolones. Although TCSs are known as regulators for bacterial adaptation to stressful conditions, which modulates β-lactam resistance in Vibrio parahaemolyticus and colistin resistance in Salmonella enterica serovar Typhimurium, there is little knowledge of their functional mechanisms underlying the development of antibiotic resistance in S. Enteritidis. Here, we systematically identified the TCS elements in S. Enteritidis SJTUF12367, revealed that the three TCSs CpxAR, PhoPQ, and GlnGL were crucial for the MDR formation in S. Enteritidis, and preliminarily illustrated the regulatory functions of CpxAR and PhoPQ for antimicrobial resistance genes. Our work provides the basis to understand the important TCSs that regulate formation of antibiotic resistance in S. Enteritidis.

KEYWORDS S. Enteritidis, two-component system, multidrug resistance, GlnGL, CpxAR, PhoPQ

Salmonella enterica serovar Enteritidis is an important foodborne pathogen causing diarrhea, fever, and abdominal cramps in humans and animals worldwide, and its
multidrug resistance (MDR) is of significant global concern (1). Among MDR isolates, the ACSSuT resistance pattern (defined as resistance to ampicillin, chloramphenicol, streptomycin [STR], sulfamethoxazole, and tetracycline [TET]) is a common characteristic in Salmonella infection (2, 3). Quinolones and cephalosporins are currently recommended for treatment of bacterial gastrointestinal diseases clinically (4). However, S. Enteritidis isolates carrying the ACSSuT profile and simultaneously co-resistant to quinolones and cephalosporins have emerged, which can complicate clinical therapy. A relatively high incidence of resistance to nalidixic acid (NAL) (95.29%), cefotaxime (CTX) (70.64%), cefepime (FEP) (58.72%) and ceftazidime (CAZ) (48.62%) has been reported among S. Enteritidis isolates with the ACSSuT profile derived from patients (5, 6). Therefore, it is of critical importance to understand the molecular mechanisms underlying the emergence of MDR and to develop therapeutic alternatives for these problematic S. Enteritidis strains.

MDR in many cases may result from potential synergies, including reduced outer membrane (OM) permeability, degraded antimicrobial agents, an altered drug target, and activated drug export. The genetic basis for resistance to β-lactam antibiotics (e.g., penicillin and extended-spectrum cephalosporins) is usually due to intrinsic or horizontally acquired β-lactamases and multiple drug exporters that can degrade and efflux commonly prescribed antibiotics (7). In addition, mutations in DNA gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) and activation of efflux pumps (e.g., AcrAB) are usually responsible for high-level quinolone resistance (8). Moreover, the genes responsible for outer membrane (OM) porins (e.g., OmpC and OmpF), which act as checkpoints to monitor the non-specific entry of many compounds, could impact membrane permeability and therefore promote an increase in tolerance to quinolones and cephalosporins in Salmonella (9, 10). Previous studies have mainly focused on the number and types of antimicrobial resistance genes (ARGs), while their modes of activation and regulation by stress response regulators such as two-component systems (TCSs) in the presence of antibiotics are rarely characterized and need to be clarified.

TCSs are crucial signal transduction system composed of a sensor histidine kinase (HK) and a response regulator (RR), by which bacteria sense and respond to environmental stresses (e.g., temperature, pH, osmolarity, and antibiotics) (11, 12). Typically, HKs sense environmental signals, autophosphorylate at the conserved histidine residue, and subsequently transfer phosphoryl to its cognate cytosolic RR at the conserved aspartate residue. The activated RR then binds to specific gene promoters to regulate their expression and therefore initiates cellular responses (11). Bacteria usually possess various TCSs, ranging from a few to over 100, and certain TCSs have been shown to contribute to antibiotic resistance formation via the regulation of ARG expression (13). For example, VbrKR in Vibrio parahaemolyticus directly senses β-lactam antibiotics and induces the expression of blaA, which encodes a functional β-lactamase to destroy or hydrolyze β-lactam antibiotics (12). EvgSA serves as the master regulator to control the expression of the efflux genes emrKY, yhiUV, acrAB, and mdfA, thus conferring MDR to Escherichia coli (14). BlrAB in Aeronomas spp. and CreBC in E. coli and Pseudomonas aeruginosa trigger the expression of ampC (which encodes β-lactamase) and thus give rise to β-lactam resistance (15–17). Therefore, interpretation of the relationship between TCSs and ARGs is of great significance to elucidate the antibiotic resistance regulatory mechanism.

The effect of TCSs on resistance formation has seldomly determined in Salmonella, and only a few reports have shown TCSs conferring antibiotic resistance in Salmonella enterica serovar Typhimurium. For instance, it is reported that CpxAR confers resistance to aminoglycosides and β-lactams by influencing the expression level of the MDR-related genes (e.g., acrB and marA) in S. Typhimurium (18). PhoPQ regulates the lipo-polysaccharide (LPS) modification loci (e.g., pbgP and ugd) and activates the TCS BasSR, which is responsible for LPS synthesis and lipid A modification to increase colistin (COL) resistance of S. Typhimurium (19). However, these studies are limited to clarifying the function of a certain TCS with susceptible S. Typhimurium isolates. Whether these regulatory mechanisms still work or not in common MDR isolates remains unknown. In addition, the TCSs CpxAR and PhoPQ are broadly conserved among many pathogenic
and nonpathogenic bacteria, but their regulons vary in different bacteria, such as S. Typhimurium, E. coli, P. aeruginosa, Yersinia pestis, and Mycobacterium tuberculosis (20–24). Previous comparative genomic studies have suggested that regulons exhibit considerable plasticity across the evolution of bacterial species (25, 26). In this regard, it has more practical significance to systematically study the contribution of TCSs, including CpxAR and PhoPQ, in MDR S. Enteritidis.

To date, most isolates of MDR S. Enteritidis from both food and clinical settings exhibit high resistance to clinically recommended antibiotics (e.g., quinolones andcephalosporins), thus limiting the clinic treatment options. Anti-signal transduction is an alternative therapy to defeat antibiotic-resistant bacteria, and thus TCSs are considered attractive antibacterial targets (27). Therefore, the systematic identification and elucidation of TCSs that contribute to MDR are crucial for the prevention and control of antibiotic resistance in S. Enteritidis. We have recently reported the complete genome of a sequence type 11 (ST11) S. Enteritidis isolate, SJTUF12367, with an extensive antibiotic resistance profile and abundant ARGs (28). S. Enteritidis ST11 strains have been reported to be a globally dominant clone, in which MDR phenotypes are common (29). The purpose of the present work was to systematically characterize the role of the TCSs in development of MDR in S. Enteritidis SJTUF12367 and other related clinical isolates through gene knockout and antimicrobial susceptibility tests and to explore the regulatory mechanisms of two important TCSs (CpxAR and PhoPQ) against quinolones and cephalosporins through interaction network analysis, quantitative reverse transcription-PCR (qRT-PCR), and membrane perturbation analysis. This work will contribute to a better understanding of antibiotic resistance mechanisms as well as a basis for combating antibiotic resistance in S. Enteritidis.

RESULTS

Functional defects in 10 pairs of TCSs resulted in an increase in S. Enteritidis susceptibility to antibiotics. Twenty-six RR genes were replaced by a flippase recognition target (FRT) site-flanked hph cassette that contains a hygromycin resistance gene as selection marker in S. Enteritidis SJTUF12367, resulting in 26 TCS deletion mutants (see Fig. S1 in the supplemental material). The growth curve of these mutants was measured in LB. As shown in Fig. 1A, compared to the wild type (WT), the ∆glnG::hph mutant exhibited delayed growth (P < 0.05) during the logarithmic phase and the ∆phoP::hph mutant displayed a little lower culture density during the stationary phase, but there was no apparent change in bacterial growth rate in the rest of the TCS mutants. It was also found that there was no significant difference in the growth rates between the ∆glnG and WT strains when 0.4% glutamine was added to the culture: i.e., the glutamine could recover the delayed growth in the ∆glnG strain, indicating the importance of GlnGL in regulation of nitrogen source synthesis (Fig. 1B).

The MICs of the WT and 26 mutants against eight classes of antibiotics were determined, which covered the most common clinical and veterinary antibiotics. The results in Table 1 show that MIC values of 10 TCS mutants were significantly altered, while those of the other 16 TCS mutants were not apparently altered (data not shown). The ∆arcA::hph mutant showed 2- and 4-fold decreases in the MICs of tetracycline (TET) and azithromycin (AZI), whereas there were 2- and 4-fold increases in the MICs of streptomycin (STR) and gentamicin (GEN), respectively, compared to the WT. In addition, 2- and 16-fold decreases in the MICs of cefotaxime (CTX) and colistin (COL), respectively, for the ∆basR::hph mutant (basR is also known as pmrB), a 4-fold decrease in the MIC of GEN for the ∆baeR::hph mutant, a 4-fold increase in the MIC of GEN for the ∆narP::hph mutant, a 2-fold decrease in the MIC of CTX for the ∆ompR::hph mutant, a 2-fold decrease in the MIC of ceftriaxone (CRO) for the ∆yehT::hph mutant, and a 2-fold decrease in the MICs of ciprofloxacin (CIP) and ofloxacin (OFX) for the ∆uvrY::hph mutant were also observed.

It was found that deletion of the gene cpxR, phoP, or glnG influenced the susceptibility of S. Enteritidis to at least three classes of antibiotics. Compared to the WT, there was a 16-fold decrease in the MIC of colistin in the ∆phoP::hph mutant and there was a 2- to 4-fold decrease in the MIC of aminoglycosides (STR, GEN, and kanamycin [KAN])
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Figure 1: Construction of TCS mutants and determination of bacterial growth curves. (A) Comparison of growth curve in the WT and mutants. Cell densities were measured as OD₆₀₀ every 30 min for 24 h. Growth curves represent the average of 3 biological replicates. (B) Bacterial growth of WT, ΔglnG, and ΔglnG::glnG strains in LB medium or LB supplemented with 0.4% glutamine (Glu); (C) schematics of phoP deletion and confirmation. (a) Schematics of phoP deletion. Black lines and parts in the gene indicate the upstream flank (UF) and downstream flank (DF). For in-frame mutagenesis, the mutation maintained a fraction of the codons of the gene. Thin arrows with names above each sequence indicate primers (Table S5) targeting corresponding positions of the sequence (not to scale). Amplicons sizes are shown in between for each pair of primers. (b) Mutant confirmation by PCR. The primers used to check phoP, phoP plus flanking sequences, and the hph cassette shown in panel a. Primers EBGNHe-5/EBGh3-3 and PR1655/PR1656 were used for determination of pKOBEG-Apra and pFLP2-Apra, respectively. Expected amplicon sizes are given in parentheses. (c) Mutations confirmed by sequencing. Only the coding sequences are shown. Red text indicates the maintained codons and the corresponding translation of amino acids.

in the ΔcpxR::hph mutant. Both ΔphoP::hph and ΔcpxR::hph strains showed 2- to 8-fold decreases in the MICs of cephalosporins (CAZ, CRO, CTX, and cefepime [FEP]) and quinolones (NAL, CIP, and OFX). Likewise, there was a 2- to 8-fold reduction in resistance to antibiotics, including pipercillin (PIP), CAZ, CRO, CTX, FEP, STR, GEN, AZI, TET, and sulfamethoxazole-trimethoprim (SXT) in the ΔglnG::hph mutant. These results were also consistent with the resistant phenotype in the hph cassette-free ΔcpxR, ΔglnG, and...
TABLE 1 Susceptibility of S. Enteritidis and its derivatives to antibiotics

| Antibiotic | Abbr | WT | ΔglnG::hph | ΔphoP::hph | ΔcpxR::hph | ΔglnG:ΔglnG | ΔphoP:ΔphoP | ΔcpxR:ΔcpxR |
|------------|------|----|------------|------------|------------|-------------|-------------|-------------|
| Piperacillin | PIP | 512 | 512 | 512 | 512 | 512 | 512 | 512 | 128 | 512 | 512 | 512 | 512 | 512 |
| Cefazolin | CAZ | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 8 | 4 | 8 | 32 | 32 | 32 |
| Ceftriaxone | CRO | 256 | 256 | 256 | 128 | 256 | 256 | 256 | 64 | 64 | 64 | 256 | 256 | 256 |
| Cefotaxime | CTX | 256 | 256 | 128 | 256 | 256 | 256 | 64 | 64 | 64 | 256 | 256 | 256 |
| Cefepime | FEP | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 4 | 4 | 8 | 32 | 32 | 32 |
| Kanamycin | KAN | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 |
| Streptomycin | STR | 256 | 256 | 256 | 256 | 256 | 256 | 256 | 128 | 256 | 64 | 256 | 256 | 256 |
| Gentamicin | GEN | 0.5 | 0.5 | 0.5 | 0.12 | 2 | 0.5 | 0.5 | 0.12 | 0.5 | 0.12 | 0.5 | 0.12 |
| Nalidixic acid | NAL | 256 | 256 | 256 | 256 | 256 | 256 | 256 | 64 | 128 | 256 | 256 | 256 |
| Ofloxacin | OFX | 0.5 | 0.5 | 0.5 | 0.5 | 0.25 | 0.5 | 0.5 | 0.25 | 0.5 | 0.25 | 0.5 | 0.5 |
| Ciprofloxacin | CIP | 0.12 | 0.12 | 0.12 | 0.12 | 0.06 | 0.12 | 0.06 | 0.06 | 0.06 | 0.12 | 0.12 |
| Colistin | COL | 8 | 8 | 0.5 | 8 | 8 | 8 | 8 | 0.5 | 8 | 8 | 8 | 8 |
| Azithromycin | AZI | 2 | 0.5 | 2 | 2 | 2 | 2 | 2 | 0.25 | 2 | 2 | 2 | 2 |
| Tetracycline | TET | 128 | 64 | 128 | 128 | 128 | 128 | 32 | 128 | 128 | 128 | 128 |
| Chloramphenicol | CHL | 256 | 256 | 256 | 256 | 256 | 256 | 256 | 16/304 | >32/608 | >32/608 | >32/608 |
| Trimethoprim- sulfonamides | SXT | >32/608 | >32/608 | >32/608 | >32/608 | >32/608 | >32/608 | >128/304 | >32/608 | >32/608 | >32/608 | >32/608 |
| Fosfomycin | FOS | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 | >512 |

*The antibiotic susceptibilities of the ΔglnG::hph, ΔphoP::hph, and ΔcpxR::hph mutants were consistent with those of the ΔglnG, ΔphoP, and ΔcpxR mutants, respectively. The ΔglnG:ΔglnG, ΔphoP:ΔphoP, and ΔcpxR:ΔcpxR strains are the complementary strains of the ΔglnG, ΔphoP, and ΔcpxR mutants, respectively. Values above or below the WT MICs are underlined or in boldface, respectively. Abbr, abbreviation.*
TABLE 2 Susceptibility of S. Enteritidis isolates to antibiotics

| Strain<sup>a</sup> | MIC (mg/L) of<sup>b</sup>: | CAZ | CRO | CTX | FEP | CIP | OFX | NAL | STR | KAN | GEN | COL | TET |
|-------------------|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| SE1 WT            |                          | 64  | 256 | 256 | 16  | 0.125 | 0.5 | 512 | 512 | >512 | 0.5 | 8   | 64  |
| Mutant ∆cpxR::hph |                          | 32  | 128 | 128 | 8   | 0.125 | 0.5 | 256 | 64  | 128  | 0.5 | 8   | 64  |
| Mutant ∆phoP::hph |                          | 16  | 64  | 128 | 8   | 0.0312 | 0.125 | 256 | 256 | 512  | 0.5 | 1   | 64  |
| Mutant ∆glnG::hph |                          | 16  | 256 | 256 | 8   | 0.125 | 0.5 | 512 | 256 | >512 | 0.5 | 8   | 16  |
| SE2 WT            |                          | 64  | 256 | 256 | 16  | 0.125 | 0.25 | 256 | 512 | 0.5  | 4   | 32  |
| Mutant ∆cpxR::hph |                          | 32  | 128 | 256 | 8   | 0.0625 | 0.125 | 128 | 64  | 128  | 0.25 | 4  | 32  |
| Mutant ∆phoP::hph |                          | 16  | 128 | 256 | 8   | 0.0312 | 0.0625 | 64  | 256 | 512  | 0.5  | 0.5 | 32  |
| Mutant ∆glnG::hph |                          | 16  | 128 | 256 | 8   | 0.0625 | 0.125 | 128 | 128 | 512  | 0.25 | 4  | 32  |
| SE3 WT            |                          | 64  | 256 | 128 | 16  | 0.125 | 0.125 | 256 | 16  | 512  | 0.5  | 2   | 4   |
| Mutant ∆cpxR::hph |                          | 32  | 128 | 128 | 16  | 0.0312 | 0.0625 | 128 | 4   | 128  | 0.125 | 2  | 4   |
| Mutant ∆phoP::hph |                          | 32  | 128 | 64  | 8   | 0.0312 | 0.0312 | 128 | 16  | 512  | 0.5  | 0.25 | 4   |
| Mutant ∆glnG::hph |                          | 16  | 64  | 256 | 8   | 0.125 | 0.125 | 256 | 16  | 512  | 0.5  | 2   | 2   |
| SE4 WT            |                          | 64  | 256 | 128 | 16  | 0.125 | 0.25 | 256 | 16  | 16   | 0.5  | 4   | 8   |
| Mutant ∆cpxR::hph |                          | 32  | 128 | 32  | 16  | 0.125 | 0.0625 | 128 | 4   | 2    | 0.25 | 4  | 8   |
| Mutant ∆phoP::hph |                          | 32  | 128 | 64  | 8   | 0.0312 | 0.0625 | 128 | 16  | 8    | 0.5  | 0.25 | 8   |
| Mutant ∆glnG::hph |                          | 16  | 128 | 128 | 8   | 0.125 | 0.125 | 256 | 16  | 16   | 0.5  | 4   | 2   |
| SE5 WT            |                          | 64  | 256 | 256 | 16  | 0.25  | 0.0625 | 512 | 512 | >512  | 0.5  | 8   | 32  |
| Mutant ∆cpxR::hph |                          | 32  | 128 | 128 | 16  | 0.125 | 0.25 | 512 | 128 | 128  | 0.25 | 4  | 32  |
| Mutant ∆phoP::hph |                          | 16  | 32  | 128 | 8   | 0.125 | 0.25 | 128 | 256 | 512  | 0.5  | 0.5 | 32  |
| Mutant ∆glnG::hph |                          | 16  | 128 | 256 | 16  | 0.125 | 0.5   | 512 | 256 | 512  | 0.5  | 8   | 16  |
| SE6 WT            |                          | 64  | 256 | 256 | 32  | 0.125 | 0.25 | 256 | 256 | 512  | 1    | 8   | 32  |
| Mutant ∆cpxR::hph |                          | 32  | 128 | 128 | 32  | 0.125 | 0.125 | 128 | 128 | 256  | 0.25 | 8  | 32  |
| Mutant ∆phoP::hph |                          | 16  | 128 | 128 | 16  | 0.0625 | 0.0625 | 128 | 256 | 512  | 1    | 0.5 | 32  |
| Mutant ∆glnG::hph |                          | 16  | 128 | 256 | 16  | 0.125 | 0.125 | 128 | 128 | 512  | 1    | 8   | 16  |

<sup>a</sup>SE1 to -6 are S. Enteritidis isolates SJTU14364, SJTU14365, SJTU14409, SJTU14745, SJTU14749, and SJTU14751, respectively.

<sup>b</sup>CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; CIP, ciprofloxacin; OFX, ofloxacin; NAL, nalidixic acid; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; COL, colistin; TET, tetracycline. Values below the parent strain’s MIC are marked in boldface.

ΔphoP mutant strains (Fig. 1C), respectively, while the complementary strains ∆cpxR::cpxR, ∆glnG::glnG, and ∆phoP::phoP complementary strains (Fig. S2) showed no obvious difference in MIC values compared with the WT (Table 1).

CpxAR, PhoPQ, and GlnGL played an important role in developing MDR in different S. Enteritidis isolates. To determine the roles of CpxAR, PhoPQ, and GlnGL in regulation of drug resistance of various S. Enteritidis isolates, six clinical MDR isolates (SE1 to -6), which exhibited resistance to cephalosporins and nalidixic acid and/or ACSSuT antimicrobial resistance profiles (Table S1), were used to construct deletion mutants with single gene deletions of cpxR, phoP, and glnG, respectively. The resulting deletion mutants were verified by PCR and sequencing (Fig. S3). The MICs for SE1 to -6 and their mutants against quinolones, cephalosporins, aminoglycosides, colistin, and tetracycline were then determined. As shown in Table 2, these wild-type (WT) isolates exhibited resistance to CAZ, CRO, CTX, FEP, and NAL. Among the 6 cpxR deletion mutants, 4 showed 2- to 8-fold decreases in the MICs of CAZ, CRO, OFX, NAL, STR, KAN, and GEN compared with their parental strains. In addition, all 6 of the phoP deletion mutants showed 2- to 16-fold decreases in the MICs of CAZ, CRO, FEP, CIP, OFX, NAL, and COL. Most of the ∆glnG::hph mutants showed 2- to 8-fold decreases in the MICs of CAZ, CRO, FEP, CIP, and TET, and a few mutants exhibited higher susceptibility to OFX, NAL, STR, or KAN than their parental strains. The changes in antibiotic sensitivity of
these mutants were consistent with those caused by cpxR, phoP, or glnG deletion in S. Enteritidis SJTU12367. Altogether, CpxAR, PhoPQ, and GlnGL all modulated resistance of S. Enteritidis to quinolones and cephalosporins, in which CpxAR also facilitated resistance to aminoglycosides, PhoPQ conferred resistance to colistin, and GlnGL mediated resistance to aminoglycosides and tetracycline. These results demonstrated that CpxAR, PhoPQ, and GlnGL contributed to the development of multiple-drug resistance in S. Enteritidis MDR isolates.

CpxAR and PhoPQ directly interacted with ARGs. To characterize the interactions between important TCSs (cpxAR, phoPQ, and glnGL) and ARGs, the ARGs of the SJTU12367 genome were collected from the 3 databases PATRIC, CARD, and NDARO and analyzed. A total of 127 ARGs (33 in PATRIC, 39 in CARD, and 55 in NDARO) were obtained, and 78 non-redundant ARGs were screened out. Finally, 55 ARGs (including cpxAR and phoPQ) showed interactions in the STRING database along with 163 functional interactions, and three densely interconnected clusters (designated C1 to C3) were obtained via Cytoscape (Fig. 2A). However, string analysis showed that glnGL had no interaction links with ARGs.

These ARGs were responsible for various resistance mechanisms, including multidrug efflux pumps (19 genes), alteration in drug targets (25 genes), enzymatic inactivation/degredation (2 genes), drug permeability restriction (1 gene), and MDR mechanisms (8 genes) (Fig. 2B). Additionally, functional enrichment analysis showed that C3 genes (phoP, phoQ,
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*cpxR, marR, acrA, mqrB, emrA, and cpxR* were enriched in the pathway of cationic antimicrobial peptide resistance (eco01503), response to stimulus (GO:0050896), drug transmembrane transporter activity (GO:0015238), and DNA binding (GO:0003677), most of which were involved in the MDR mechanisms (data not shown).

To explore the ARGs’ correlation with *cpxAR* and *phoPQ*, the extended network with C3 genes was analyzed. The functional partners of C3 genes were classified into class I and II based on their functions via STRING clusters (Fig. 2C and Table S2). The genes in class I (CL:46) consisted of rpo, rps, and rpl operons, which encoded RNA polymerase (RNAP) and the 30S and 50S ribosomal subunits, respectively, and were responsible for ribosome assembly. Another observation in class II (CL:3574) showed a high number of interconnections among efflux pump genes (*acrAB, acrD, macB, and emrAB*), global regulator genes (*marA and cpxR*), and the *marR* operon repressor gene (*marR*), which were enriched in the pathway of response to antibiotic (GO:0046677) and transmembrane transporter activity (GO:0015238), and DNA binding (GO:0003677), most of which were significantly different among all mutants. However, deletion of *cpxR* or *phoP* resulted in higher expression levels of *ompC and ompF* genes than the WT (P < 0.05) after 10 h, which indicated that the homeostasis of bacterial envelope was disturbed.

**Deletion of *phoP/cpxR* resulted in an increase in cell membrane permeability.**

The relative mRNA expression of a series of ARGs among WT and Δ*phoP/ΔcpxR* cells was determined via quantitative reverse transcription-PCR (qRT-PCR). In this work, ceftazidime (CAZ) and nalidixic acid (NAL) were used as the representatives of quinolones and cephalosporins, respectively. The subinhibitory concentrations of 2 mg/L CAZ (1/16 MIC) and 32 mg/L (1/8 MIC) NAL were selected because all strains could grow at these concentrations, and the population density of Δ*cpxR/ΔphoP* mutants was able to reach a WT level (Fig. S4).

The porins OmpA, OmpC, OmpD, and OmpF were abundant in bacterial outer membrane, among which OmpC and OmpF were linked directly to ciprofloxacin resistance (34), while OmpA was a large porin that allowed for slow diffusion of molecules at a rate 50 times lower than that of OmpF (35). As shown in Fig. 3A, the transcriptional levels of the *ompA, ompC, ompD, and ompF* genes were measured at the bacterial exponential growth phase (2 h) and the early stationary phase (8 h or 10 h) under the unstressed condition. Compared to WT cells, the transcriptional levels of *ompC* and *ompA* genes were not significantly different among all mutants. However, deletion of *cpxR* or *phoP* resulted in higher expression levels of *ompD* and *ompF* genes than the WT (P < 0.05) after 10 h, which indicated that the homeostasis of bacterial envelope was disturbed.
To test the hypothesis that the ΔcpxR/ΔphoP mutants were more permeable to small-molecule antibiotics than the WT, we treated bacterial cells with subinhibitory concentrations of ceftazidime (2 mg/L) or nalidixic acid (32 mg/L). As expected, the differences in the expression of porins between the WT and mutants were enlarged after adding antibiotics (Fig. 3A). The expression levels of the *ompC*, *ompD*, and *ompF* genes were significantly (*P* < 0.01) higher in ΔcpxR/ΔphoP mutants than in the WT at the early stationary phase. The expression level of the *ompA* gene did not apparently differ

**FIG 3** CpxAR and PhoPQ modulate membrane permeability. (A) Expression analysis of outer membrane porin genes in ΔphoP, ΔcpxR, and WT cells. Shown are results from qRT-PCR analysis of the mRNA expression levels of the *ompA*, *ompC*, *ompD*, and *ompF* genes in cells untreated (top), treated with 32 mg/L of NAL (middle), and treated with 2 mg/L of CAZ (bottom). All points were expressed relative to the untreated WT at 2 h, represented by the dotted gray line. Significance analysis was determined by the Holm-Sidak method. Error bars represent 95% confidence intervals. (B) NPN uptake assay. Error bars indicate the standard deviation of the results from 5 technical replicates. *, *P* < 0.05; **, *P* < 0.01.
among all strains under NAL pressure, but expression was significantly ($P < 0.001$) higher in mutants than the WT under CAZ stress for 8 h.

Permeabilization activity against membranes of *S*. Enteritidis wild-type and Δ*cpxR*/Δ*phoP* mutants was determined using 1-N-phenylnaphthylamine (NPN) uptake assays (36). As shown in Fig. 3B, the fluorescence intensity of NPN was not significantly changed among WT cells not under stress or with CAZ and NAL added, indicating that subinhibitory concentrations of antibiotics did not destroy the OM permeability of WT. In addition, the fluorescence intensities of WT, Δ*phoP*, and Δ*cpxR* cells were similar without antibiotic treatment. However, compared to WT cells, the NPN fluorescence intensity was significantly increased in Δ*cpxR* and Δ*phoP* cells after adding antibiotics and was especially markedly increased in the Δ*cpxR* and Δ*phoP* cells under the ceftazidime condition ($P < 0.01$). The difference between NAL- and CAZ-treated cells could arise from severe membrane damage by β-lactam-mediated perturbation of peptidoglycan biosynthesis, which led to destabilization or lysis of the cellular envelope. Overall, CpxAR and PhoPQ could maintain and modify bacterial membrane permeability by regulating expression of OM porins and result in an increase in the resistance of *S*. Enteritidis to quinolones and cephalosporins.

**CpxAR and PhoPQ strengthened the specific efflux pump activity.** To characterize the role of PhoPQ and CpxAR in the function of efflux pumps, qRT-PCR was carried out with or without antibiotic to test the transcription characteristics of 4 families of efflux pump genes, including the resistance-nodulation-cell division (RND) family pumps (*acrA*, *acrD*, and *mdsA*), the major facilitator superfamily (MFS) pump (*emrA*), the multi-drug and toxic compound extrusion (MATE) family pump (*mdtK*), and the ATP-binding cassette (ABC) family pump (*macB*). After an incubation for 10 h with nalidixic acid, Δ*phoP* mutant cells were shown to have significantly reduced expression levels of the *macB* and *mdtK* genes, while the Δ*cpxR* mutant had lower expression levels of the *acrA* and *acrD* genes ($P < 0.001$) than the WT (Fig. 4A and B). After ceftazidime treatment for 8 h, there was no significant alteration in the expression levels of *macB* and *mdtK* genes in the Δ*cpxR* mutant compared to the WT, whereas the expression levels of *acrA* in the
of the \( CpxR \) mutant and \( macB \) in the \( \Delta \text{phoP} \) mutant were significantly decreased \( (P < 0.01) \). In addition, at the early stationary stage of cultivation after antibiotic treatment, extremely increased expression levels of the \( \text{emrA} \) gene in \( CpxR \) and \( \text{phoP} \) cells were achieved compared to those of WT cells. These results demonstrated that \( \text{phoP} \) and \( CpxR \) promoted bacterial resistance to NAL and CAZ through regulation of the expression of efflux pumps. In this case, PhoP played a positively regulatory role in MacB and MdtK pumps, while CpxR had an obvious effect on activation of RND pumps; in contrast, the partial abnormalities of aforementioned efflux pumps might be neutralized or compensated via overexpression of other pumps (e.g., \( \text{EmrA} \)) under antibiotic pressure at subinhibitory concentrations.

**CpxAR and PhoPQ modulated the expression of specific global regulatory factors.** As presented in the C3 extended network (Fig. 2C), the global regulatory factors were key nodes in CpxAR- and PhoPQ-mediated regulation of efflux pump genes. qRT-PCR results showed that there were 2- to 4-fold increases in the expression levels of the \( \text{marA} \), \( \text{soxS} \), and \( \text{ramA} \) genes and a 4-fold decrease in the expression level of \( \text{crp} \) gene in NAL-treated cells, whereas no significant difference was observed in CAZ-treated cells compared to untreated WT cells at 2 h (Fig. 5). When cells were treated at the early stationary stage, the expression level of \( \text{ramA} \) was significantly lower in the \( \Delta \text{cpxR} \) mutant, but in the \( \Delta \text{phoP} \) mutant, \( \text{ramA} \) expression was similar to or higher than that in the WT cells. In addition, the expression level of the \( \text{crp} \) gene in \( \Delta \text{phoP} \) mutant was significantly \( (P < 0.001) \) increased, which had not been identified in previous studies under antibiotic conditions, indicating the negative regulation of PhoP on \( \text{crp} \) expression. Furthermore, the expression level of \( \text{marA} \) gene was increased and higher
in all mutant cells than in the WT, indicating that MarA possessed the compensatory function to overcome subinhibitory concentrations of antibiotics. Therefore, the lack of CpxR not PhoP would impair the global regulator of RamA, thereby indirectly and positively regulating the function of RND efflux pump; correspondingly, PhoP acts in a crp-dependent manner.

There existed a compensatory function between PhoP and CpxR. The expression levels of the phoP and cpxR genes in WT and mutants were determined. Although the expression level of phoP showed no significant difference among ΔcpxR, NAL-treated ΔcpxR (ΔcpxR+NAL) cells, and CAZ-treated ΔcpxR (ΔcpxR+CAZ) cells, a comparatively higher expression level of phoP was found in ΔcpxR cells than in WT cells (P, 0.05) (Fig. 6). Similarly, the expression level of cpxR in ΔphoP cells was significantly higher than those in WT cells. In addition, significantly higher expression levels of cpxR in ΔphoP+NAL or ΔphoP+CAZ cells than untreated ΔphoP cells were also observed (except for NAL treatment at 10 h). These data indicated that there was a compensation mechanism between phoP and cpxR functions, which might partially account for the antibiotic pressure fitness of S. Enteritidis.

**DISCUSSION**

In the present study, a large-scale mutational analysis was conducted on MDR S. Enteritidis to explore the role of TCSs in the development of antibiotic resistance. Our results revealed that 38.46% of the TCS pairs were involved in antibiotic resistance of S. Enteritidis. Among these TCSs, BarA/UvrY, NarQP, and ArcBA, previously known as regulators for metabolic control (37–39), were found to facilitate resistance development. Additionally, the absence of yehT and basR resulted in an increase in susceptibility of S. Enteritidis to ceftriaxone and colistin, respectively, in agreement with published studies where overexpression of yehT caused a 2-fold increase in MIC of β-lactams and mutation in PmrAB resulted in a decrease in resistance to colistin (19, 40). However, in the present study, the mutants with deletion of baeR and ompR exhibited increased susceptibility to gentamicin and cefotaxime, respectively, whereas overexpression of baeR was previously reported to lead to increased resistance to ceftiofur and cefotaxime, and alteration of EnvZ/OmpR contributed to ertapenem and nalidixic acid resistance in E. coli (41–43). Thus, there were differences in the effects of TCS orthologs on bacterial resistance.
The TCSs CpxAR, PhoPQ, and GlnGL were shown to play important roles in MDR of S. Enteritidis SJ12367, which was observed in multiple S. Enteritidis isolates, indicating the universal contribution of these TCSs to the MDR phenotype of S. Enteritidis serovars. It was previously reported that the absence of phoPQ led to attenuated expression of mltD1 and sI and increased β-lactam influxes in Stenotrophomonas maltophilia (44), and inactivation of CpxR resulted in higher susceptibility to aminoglycosides and β-lactams of S. Typhimurium (18). In our findings, phoP and cpxR mutants additionally exhibited higher susceptibility to nalidixic acid, ciprofloxacin, and ofloxacin with a drastic decrease (up to 8-fold) in MIC, suggesting a more extensive influence of these TCSs on antibiotic resistance. GlnGL (also called NtrBC) was a key regulator in response to nitrogen source and nitrogen availability via activation of expression of glnA, which encoded a glutamine synthetase (GS) (45). Recently, a few clues in susceptible strains showed that the absence of ntrBC in E. coli and glnA in Salmonella enterica serovar Typhi could increase their susceptibility to aminoglycosides and ciprofloxacin, respectively (46, 47). In this study, a phenotype in S. Enteritidis glnG mutants of relative susceptibility to quinolones and aminoglycosides was observed, and the absence of glnG also led to an increase in susceptibility to cephalosporins and tetracycline, suggesting a greater influence of glnG on MDR S. Enteritidis than on susceptible strains. Overall, these TCSs were crucial for development of multidrug resistance in S. Enteritidis.

Gene network analysis is one of the reliable approaches that provide new insights into biological information of antimicrobial resistance mechanisms. In this work, the ARGs were placed into three clusters. The genes in cluster C1 (e.g., gyrA, rpoB, and rplC) were responsible for alteration in drug targets (48–52); however, there was only one single-base mutation in GyrA, D87G, found in the SJ12367 genome. The genes in cluster C2 (clsA, clsC, and pgsA) were enriched in the phospholipid biosynthesis pathway (KW-0594) and responsible for synthesis of cardiolipin and phosphatidylglycerol synthesis, whose loss could be compensated for by other anionic phospholipids (53). The genes in cluster C3 were deemed essential for MDR phenotype, and the associations among PhoPQ, CpxAR as well as global regulators were found in the C3 extended network, which indicated a coupling of signaling of CpxAR or PhoPQ in modulating the global regulators and relevant genes of the efflux pump and OM porin. Unfortunately, no interaction links were found between glnGL and ARGs. Previous studies reported that glutamine was responsible for synthesis of a poly-α-L-glutamine layer associated with the cell wall, and inhibition of GS activity enhanced the susceptibility to penicillin in Streptococcus pneumoniae and methicillin in Staphylococcus aureus (54, 55). In addition, the E. coli TCS GlnGL could govern transcription of relA/spoT genes and thereby led to elevated level of guanosine tetra- and pentaphosphate (ppGpp). In Vibrio cholerae, accumulation of ppGpp led to a suppressed central metabolism and iron transport system, resulting in reduced oxidative stress and increased antibiotic resistance (tetracycline, erythromycin, and chloramphenicol) (56, 57). Altogether, we speculated that S. Enteritidis GlnGL could activate expression of GS and ppGpp, which were involved in production of cell envelope constituents and regulation of intracellular redox balance, therefore conferring antibiotic resistance.

The regulatory effects of CpxAR and PhoPQ on multiple ARGs were demonstrated in this study. Bacteria could survive antibiotic exposure by expelling antibiotics through efflux pumps and blocking OM porin-mediated antibiotic influx (58). The global regulators RamA, SoxS, and MarA could modulate expression of efflux pumps and OM porins in Salmonella (59, 60). In this work, the absence of cpxR/phoP caused an increase in the expression level of OM porins and in membrane permeability as well as a decrease in expression level of specific efflux pumps and their regulatory factors, resulting in influx of a quantity of antibiotic molecules. Previous studies showed that the expression of OM porins STM1530 and OmpD was regulated by CpxAR to promote ceftriaxone resistance of S. Typhimurium (61). In E. coli, overproduction of CpxR was found to confer resistance to β-lactams in an acrB-free background (40). P. aeruginosa CpxAR was found to enhance quinolone resistance by activating expression of the mexAB-oprM efflux pump, while the
absence of CpxR led to a *Klebsiella pneumoniae* phenotype susceptible to β-lactams and chloramphenicol (24, 62). Therefore, the regulatory function of CpxAR on efflux pumps and OM porins was universal across different species. On the other hand, it was proposed that PhoPQ could activate the putative ABC transporters in *P. aeruginosa* to extrude intracellular tetracycline (63), and the MdtK pump could expel ciprofloxacin into the periplasmic space in *E. coli* (64). To our knowledge, there were no direct clues to the resistance regulatory function of PhoPQ in *S. Enteritidis*. This study clarified the correlation between PhoPQ and efflux pumps by demonstrating the upregulatory effect of PhoPQ on the transcription level of the ABC-type family and MATE family pump genes (macB and mdtK). In addition, PhoP was reported as a transcriptional activator of *crp* in *Yersinia pestis* (65) and the loss of *crp* in *S. Typhimurium* could enhance resistance to fluoroquinolones by reducing permeability and elevating efflux of fluoroquinolones (33). Given the increased level of expression of *crp* in ΔphoP cells, we proposed that the absence of PhoP could derepress expression of *crp* and affect the function of multiple ARGs. In any case, CpxAR and PhoPQ in *S. Enteritidis* could modulate expression of the ARGs that encoded OM porins, efflux pumps, and global regulators, conferring resistance to quinolones and cephalosporins.

Previous studies proposed that there was a feedback mechanism employed to regulate expression of AcrAB-ToIC and other functional efflux pumps by RamA and MarA (30, 60). Accordingly, a variety of compensatory pathways that improve fitness under the subinhibitory antibiotic pressure were presented in this study. Activation of EmrA pump in the absence of phoP or cpxR cells could be attributed to this feedback mechanism, which compensated for the loss of other pumps’ efflux function. In addition, it has been demonstrated that CpxR might regulate the colistin susceptibility of *S. Typhimurium* through PmrAB and PhoPQ (66). In this work, CpxR and PhoP showed cross-interaction in gene interaction networks, and qRT-PCR analysis further confirmed a compensatory expression of these two regulator genes, indicating a partial functional redundancy between PhoPQ and CpxAR. Nevertheless, these fitness mechanisms were not sufficient to completely make up for the sensitive phenotype caused by phoP/cpxR deletion, despite the adaptation of the ΔphoP and ΔcpxR mutants to antibiotics.

In conclusion, three TCSs, CpxAR, PhoPQ, and GlnGL, were identified as important regulators for MDR formation in *S. Enteritidis*. Deficiency in each of these TCSs results in a significantly reduced resistance to at least three classes of antibiotics, and this change was universal in *S. Enteritidis*. CpxAR and PhoPQ conferred resistance to quinolones and cephalosporins by altering expression of the genes responsible for drug efflux and drug entry, as well as those that caused significant changes in the global regulators. These TCSs were therefore considered potential targets in synergistic antibiotic therapy of MDR *S. Enteritidis*. In-depth characterization of crucial ones (e.g., GlnGL) that operate through gene regulation to uncover multiple cellular pathways for MDR is ongoing in our laboratory.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. Enteritidis* isolates (*n* = 7) were recovered from stool samples of patients with diarrhea during 2007 to 2017 in Shanghai and Henan, China, that were provided by the Shanghai Municipal Center for Disease Control and Prevention and reported in our recent papers (28, 67). These isolates belonged to ST11 (the predominant ST in *S. Enteritidis*) and exhibited the frequent antimicrobial resistance profile of AMP-SXT-NAL-CAZ-CRO-CTX-FEP (see Table S1 in the supplemental material), among which *S. Enteritidis* SJTUF12367 had the widest drug resistance profile (ACSSuT-NAL-KAN-CAZ-CRO-CTX-ceftiofur [TIO]-FEP-fosfomycin [FOS]). These strains were cultivated in lysogeny broth (LB) or on LB agar supplemented with or without appropriate antibiotics.

**Construction of gene deletion and complementation strains.** The TCS elements of the SJTUF12367 genome were identified via HMMER software and the Pfam database (Table S3 and Table S4). TCS gene deletion and complementation were performed as described previously (68). Briefly, TCS genes were first replaced by an FRT site-flanked *hph* cassette, which was a hygromycin resistance gene that was used as the resistance selection marker to construct homologous recombinant fragments and conferred resistance to hygromycin of TCS deletion strains. The cassette was then eliminated via Flp-FRT recombination to obtain markerless in-frame indel mutants. For the construction of complementary strains, the full-length regulator protein gene and its ribosome binding site were cloned into the pBAD33 vector with an arabinose-inducible

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promoter and then transformed into the corresponding mutants. The resulting strains were confirmed by PCR and DNA sequencing. The plasmids and primers used are shown in Table S1 and Table S3, respectively.

Antimicrobial susceptibility testing was conducted on S. Enteritidis isolates using the standard agar dilution method recommended by the CLSI (69). The antibiotics used included third- and fourth-generation (broad-spectrum) cephalosporins (ceftizidime, ceftriaxone, cefotaxime, cefotuor and cefepime) as well as nalidixic acid, ofloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole, ampicillin, gentamicin, streptomycin, kanamycin, azithromycin, tetracycline, and chloramphenicol. All antibiotics were purchased from Sigma, Inc. E. coli ATCC 25922 was used as a quality control for the MIC value determination.

Construction of the gene interaction network. ARGs of the SJTU12367 genome were collected from three databases, including the Pathway-systems Resource Investigation Center (PATRIC; https://www.patricbrc.org/), Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/), and National Database of Antibiotic Resistance Organisms (NDARO; https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/) (70). The interacting partner data were curated from the STRING database with the medium confidence scores (more than 0.4). The gene interaction network was visualized by Cytoscape. The Cytoscape tools MCODE and NetworkAnalyzer were used for identification of the highly interacting nodes (clusters) and computing the topological parameters for networks, respectively.

Membrane perturbation assays. The 1-N-phenyl-2-napthylamine (NPN) uptake assay was performed to determine the outer membrane permeability (36). NPN was added to the culture containing $2 \times 10^9$ CFU/mL S. Enteritidis wild-type and ΔpapRΔphoP cells (final NPN concentration of 12.5 $\mu$M) and incubated for 15 min with various concentrations of antibiotics. The fluorescence emission intensity was recorded ($A_{em} = 340$ nm; $A_{ex} = 420$ nm; gap width, 1 mm) using an Infinite 200 PRO microplate reader (Tecan, Switzerland).

RNA isolation and qRT-PCR assays. The cultures of exponentially growing cells were equalized to an optical density at 600 nm (OD$_{600}$) value of 0.2. Volumes of 160-$\mu$L bacterial suspensions were then added to 3.84 mL LB, to which had been preadded subinhibitory concentrations of antibiotics to reach final concentrations of 32 mg/L (1/8 MIC) nalidixic acid (NAL) and 2 mg/L (1/16 MIC) ceftazidime (CAZ) (Fig. S4) (33). Samples were taken after incubation at 2 h and 8/10 h. RNA was extracted using TRIzol reagent (Invitrogen) as described by Huang et al. (48). Removal of residual genomic DNA and cDNA synthesis was conducted using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). RNA integrity was confirmed by electrophoresis. The primers used are shown in Table S5. qRT-PCR was run in triplicate and amplified using the TB green Premix II reagent (TaKaRa). The relative gene transcriptional level was determined using the threshold cycle (2$^{-\Delta \Delta CT}$) method (71) and converted into the log$_2$ value. The expression of 165 rRNA was used as an internal reference. qRT-PCR data analysis was undertaken by a two-way analysis of variance (ANOVA) and Holm-Sidak’s multiple-comparison test via the Prism8.0.1 program, and all points were normalized relative to the untreated WT at 2 h.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.8 MB.
FIG S2, TIF file, 0.3 MB.
FIG S3, TIF file, 0.5 MB.
FIG S4, TIF file, 0.6 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.03 MB.
TABLE S4, DOCX file, 0.02 MB.
TABLE S5, DOCX file, 0.02 MB.

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We declare no conflict of interest.
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