Infection and Drug Resistance

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ORIGINAL RESEARCH

GLO1 Contributes to the Drug Resistance of *Escherichia coli* Through Inducing PER Type of Extended-Spectrum β-Lactamases

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Background: *Escherichia coli*-associated antimicrobial resistance (AMR) issue so far needs urgent considerations. This study aims to screen the potent genes associated with extended-spectrum β-lactamases (ESBLs) in drug-resistant *Escherichia coli* and elucidate the specific drug-resistant mechanism.

Methods: Clinical ESBLs-EC samples were obtained based on the microbial identification, and the whole genome was sequenced. In combination with the significantly enriched pathways, several differently expressed genes were screened and verified by RT-PCR. Furthermore, through knocking out glyoxalase 1 (GLO1) gene and transfecting overexpressed plasmids, the potential relationship between GLO1 and ESBLs was then investigated. Lastly, the concentrations of β-lactamases in bacteria and supernatant from different groups were examined by enzyme-linked immunosorbent assay (ELISA).

Results: After successful isolation and identification of ESBLs-EC, the whole genome and eighteen differential metabolic pathways were analyzed to select differently expressed genes, including add, deoD, guaD, speG, GLO1, VNN1, etc. RT-PCR results showed that there were no differences in these genes between the standard bacteria and susceptible *Escherichia coli*. Remarkably, the relative levels of four genes including speG, Hdac10, GLO1 and Ppcdc were significantly increased in ESBLs-EC in comparison with susceptible strains, whereas other gene expression was decreased. Further experiments utilizing gene knockout and overexpression strains confirmed the role of GLO1. At last, a total of 10 subtypes of β-lactamases were studied using ELISA, including BES-, CTX-M1-, CTX-M2-, OXA1-, OXA2-, OXA10-, PER-, SHV-, TEM-, and VEB-ESBLs, and results demonstrated that GLO1 gene expression only affected PER-β-lactamases but had no effects on other β-lactamases.

Conclusion: SpeG, Hdac10, GLO1 and Ppcdc might be associated with the drug-resistant mechanism of *Escherichia coli*. Of note, this study firstly addressed the role of GLO1 in the drug resistance of ESBLs-EC, and this effect may be mediated by increasing PER-β-lactamases.

Keywords: GLO1, bacterial drug resistance, extended-spectrum β-lactamases, *Escherichia coli*, PER type

Introduction

Acute respiratory infection is currently one of the top ten global public health threats that humans are facing, and the resulting antimicrobial drug resistance (AMR) is becoming more serious.[1] One of the most common causes is the casual application of antimicrobials, which has apparently not been a worldwide concern until now.[2] Especially, the global pandemic of Coronavirus disease 2019 (COVID-19) highlights the weakness of most of countries in preventing and responding to health emergencies, in particular for respiratory tract infections.[3,4] Therefore, there is no doubt that we should give sufficient attention and research support to this issue. Of interest, the problem of AMR derived from acute infections as well as other severe infections needs urgent considerations and resolutions. Given the fact that *Escherichia*

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coli is one of the most common pathogens of nosocomial infections especially in pneumonia, abdominal infections, uncomplicated cystitis, and bacteremia,[5,6] *Escherichia coli*-associated AMR research is under active investigation so far.

Among various regulatory mechanisms, an increasing body of evidence have clearly defined the production of extended-spectrum β-lactamases (ESBLs) as one of the main mechanisms of inducing drug resistance in *Escherichia coli*.[7,8] β-lactamase is a class of enzymes, which can hydrolyze the β-lactam ring of some antibiotics. This is how β-lactamase mediates the resistance of bacteria to these antibiotics. However, there currently exist different types of β-lactamases, which make this issue more complex.[9,10] For example, Koirala et al revealed the prevalence of CTX-M type of β-lactamases producing multidrug resistant (MDR) *Escherichia coli* in Klebsiella pneumoniae patients.[10] Therefore, the research on β-lactamase-related mechanisms of ESBLs-EC has important clinical significance.

On the other hand, glyoxalase I (GLO1) has been extensively studied in the context of microorganisms, such as bacteria, yeast, and protozoa, etc.[11–13] Originally, GLO1 has been found to catalyze the condensation of methylglyoxal. In the expanded functional sense, GLO1 might affect protein modification, oxidative stress and apoptosis, because its detoxified methylglyoxal is a cytotoxic byproduct of glycolysis.[14] Newly published studies about GLO1 focused on unique structural characteristics, which can theoretically give novel indications for exploring other new functionalities. Recently, Chirgadze et al demonstrated that human GLO1 belongs to a subfamily A, whereas GLO1 of *Staphylococcus aureus* should be a member of subfamily B. Of interest is that the newly found differences between two subfamilies could guide the design of new drugs against *Staphylococcus aureus*.[11] Moreover, Bythell-Douglas et al recently identified the crystal structure of GloA2 from *Pseudomonas aeruginosa*, and further revealed that GloA2 can serve as a hydrolase besides its GLO1 activity.[15] Later in 2018, structural features as well as functional activities of GLO1 protein family were comprehensively reviewed by Kargatov and Chirgradze, characterizing GLO1 family as "life-essential proteins" and addressing their functional significance.[14] In addition to bacteria, GLO1 also exerts important physiological functions in yeast with respect to stress response due to the fact that *Saccharomyces cerevisiae* has the sole gene encoding GLO1.[12] Collectively, GLO1 is highly involved in the pathogenicity of microorganisms and can be a promising target to be focused on.

Our previously published study identified 1553 differentially expressed proteins (DEPs) between ESBLs-EC and non-ESBLs-EC based on proteomics analysis, and 606 and 459 altered metabolites in positive and negative ion modes through an untargeted metabolomics assay,[16] suggesting that these DEPs and differential metabolites may play an important role in the antibiotic resistance of ESBLs-EC. Intriguingly, the purine metabolism pathway was enriched in ESBLs-EC. Further correlation analysis of metabolomics and proteomics data established a robust association between DEPs and differential metabolites, providing the theoretical basis for the mechanistic study of antibiotic resistance of ESBLs-EC at the metabolite and protein levels. Therefore, this study was aimed at revealing the potent genes involved in the regulation of drug resistance, investigating the specific regulatory mechanism how differentially expressed genes (DEGs) regulate drug resistance through certain type of β-lactamases produced by ESBLs-EC, and further providing some novel targets for future antibiotic development.

Materials and Methods

Clinical Bacterial Specimen Collection and Cultivation

Original clinical specimens including patient phlegm, urine, blood, or secretions were randomly collected from January to June 2018 from the Second Hospital of Jilin University, and bacterial samples were isolated using the filter paper method. These specimens were identified as *Escherichia coli* by VITEK®2 Compact (BioMérieux, Hazelwood, MO, USA), and then divided into the experimental group (n = 10) and the control group (n = 10). The same amount of each sample was isolated, and 10 pieces of samples from each group were mixed as one specimen. After each group was fully mixed, each specimen was randomly divided into three parts for following experiments. The rest of strains was stored at −80°C for future use. All the patients signed informed consent forms for the experimental study. All experimental procedures were performed in accordance with the Guidelines for the Collection and Application of Human Related Specimens of the Second Hospital of Jilin University and approved by the Ethics Committee of the Second Hospital of Jilin University. Detailed information and procedures have been described in the previous study.[16]

Escherichia coli Culture

Frozen bacterial samples were incubated by 0.9% physiological saline and placed in a 37°C water bath for 10 min. Then, 1 mL bacterial solution from each specimen was taken, placed in LB broth culture medium, incubated in a 37°C shaking incubator (Donglian Ha'er Instrument Manufacture Limited Company, Beijing, China) for 16 h at the speed of 220 rpm/min. After shaking, 200 μL bacterial solution was prepared for enzyme verification, and the rest of the bacterial solution were centrifuged, washed, and frozen for future use. In this study, the ATCC25922 strain is used as the reference standard strain. The strain from the control group (susceptible *Escherichia coli*) was designated as the No. 0 strain, and the other strain from the experimental group (ECBLs-EC) was designated as the No. 11 strain.

Verification of the Production of Extended-Spectrum β-Lactamases in *Escherichia coli*

The bottom of the MH plate was divided into quarters. Then, 1 mL bacterial liquid was taken, mixed to 0.5 MCF (McFarland standards) with a turbidimetric tube, and placed on the LB plate. The test strips of ceftazidime, ceftazidime/clavulanic acid, cefotaxime, cefotaxime/clavulanic acid (J&K Chemical Ltd., Shanghai, China) were placed in the center of each area. The plate was placed in a 37°C incubator for 12 h, and the diameter of the inhibition ring was measured respectively. When the diameter of the antibacterial circle compounded with clavulanic acid is 5 mm or more than that without clavulanic acid, it can be judged as the *Escherichia coli* strain producing ESBLs. The above operating methods and experimental results refer to the 2014 standard of the Clinical Laboratory Standardization Institute (CLSI).

Escherichia coli Total DNA Extraction and Quality Inspection

The total DNA of *Escherichia coli* was extracted using a complete gold bacteria extraction kit EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, Beijing, China), and the detailed procedures are as follows. A volume of 1 mL of the cultured bacteria was processed by adding 1000 μL of LB11 and 20 μL of proteinase, and shaken until the cells were completely suspended. Then, bacteria were incubated at 55°C for 15 min to obtain a clear solution. To remove RNA, 20 μL RNase was added to bacteria and together incubated for 2 min, and then 400μL of BB11 was added for 30 sec. After centrifugation, 500μL of CB11 was added, and then 500μL of WB11 containing ethanol was used to wash. Afterwards, the spin column was placed in a clean centrifuge tube, and 50–200 μL of preheated EB (60–70°C) was added. The eluted DNA was collected and stored at −20°C.

As for the inspection of isolated DNA quality, 1% agarose gel was used to check DNA integrity, and a Qubit fluorometer (ThermoFisher, Waltham, Massachusetts, United States) was used for DNA quantification. Electrophoresis was applied at the voltage of 200 V for 30 min.

Construction of *Escherichia coli* DNA Library

For the fragmentation of genomic DNA, QubitTM dsDNA HS Assay Kit (ThermoFisher, Waltham, Massachusetts, United States) was used to accurately quantify the genome concentration to determine the total amount of DNA, which are needed for the library construction. The library with an insert length of about 500 bp was prepared, and the initial amount of DNA was about 500 ng. The initial DNA was diluted to 130 μL with Elution Buffer and put it into a 0.5 mL Covaris DNA disruption tube. 1x Hieff NGSTM DNA Selection Beads (Yeasen BioTechnologies Ltd., Shanghai, China) were utilized to concentrate and recover the broken DNA fragments. Afterwards, the library was constructed following the instructions of NEB Next® UltraTM DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, United States). At last, the library size was checked by electrophoresis using 2% agarose gel, the concentration was measured using Thermo Qubit 4.0 (ThermoFisher, Waltham, Massachusetts, United States), and the distribution was detected through Agilent Technologies 2100 DNA 1000 Kit (Shanghai Furi Technology Co.,Ltd., Shanghai, China).

Whole-Genome Sequencing and Mapping of Drug-Resistant Bacteria

Whole-genome mapping and sequencing of the No. 11 drug-resistant strain was performed based on the framework of the Illumina™ second-generation sequencing platform. To acquire relatively accurate and valid data, the quality of original sequencing data was evaluated by FastQC, and the shearing on Illumina sequencing data was performed through

Trimmomatic. Then, SPAdes was applied to assemble the second-generation sequencing data, and GapFiller was used to supplement GAP on the coating obtained by splicing. PrInSeS-G was used for sequence correction, in order to correct editing errors and indels of small fragments during the splicing process. Gene annotation was analyzed to obtain GO function annotation and KEGG annotation information. Genome advanced annotation was performed as follows: NCBI Blast+ was applied to compare the gene and protein sequences with VFDB, CARD, PHI-base and other databases, and obtain function comment information. HMMER3 was used to compare gene and protein sequence with CAZy database to get its function annotation information; ProtCamp for protein subcellular localization analysis; and PhiSpy for prophage prediction analysis.

Real-Time PCR to Confirm Differently Expressed Genes of Drug-Resistant Bacteria

The cultured ATCC25922 strain, No. 0 susceptible strain, and No. 11 resistant strain were collected, and completely lysed by 1 mL TRIzol reagent (Invitrogen, Waltham, Massachusetts, United States). 200 μL of chloroform and isopropanol (Sangon Biotech, Shanghai, China) was gradually added, and 500 μL 75% ethanol was used to wash RNA. The precipitate was dissolved with 20 μL DEPC-H_2O, and OD_{260} and OD_{280} values were measured, and the RNA concentration was calculated. cDNA synthesis using random primers purchased from company were performed following the instruction of First strand cDNA-synthesis kit (ThermoFisher, Waltham, Massachusetts, United States). Gene expression was determined by real-time quantitative PCR (RT-qPCR) by using Takyon PCR SYBR Green mastermix Blue (Eurogentec, Seraing, Belgium). The CFX96 Real-Time PCR Detection System was used for amplification with the following settings: 10 min of initial denaturation at 95°C, followed by 40 cycles of amplification (15 s 95°C denaturing and 1 min 60°C for annealing). Validated primer sequences are shown in Table 1. Gene expression data was analyzed with the standard curve method and data was normalized to 16S as a reference gene.

Construction of Specific GLO1 Knockout Strain

In this study, the knockout of GLO1 gene by suicide plasmid method was performed on resistant ECBLs-EC, and No. 11 strain was used here. The specific steps are described as follows. To obtain the donor bacterium β-2155/pCVD442-Δglo::Cm which was initially designed for this study, pCVD442-Δglo::Cm was transferred into *Escherichia coli* β-2155 using electroporation, and then positive clones were screened on chloramphenicol plates, called *Escherichia coli*-11/pCVD442-Δglo::Cm. The single clones in which the GLO1 gene is replaced by the Cm resistance gene were selected and named *Escherichia coli*-11/Δglo::Cm. In the meanwhile, targeting plasmid and donor bacteria were prepared based on specific primer sequences. Then the positive clones were screened and the targeting plasmid was constructed. The targeting vector was transferred to *Escherichia coli* β-2155 by electroporation, and plated in the medium containing 100 μg/mL ampicillin and 0.5 mM DAP to select positive clones, named β-2155/pCVD442-Δglo::Cm. The recipient bacteria were plated to form a single clone, and the β-2155/pCVD442-Δglo::Cm clone was amplified. 500 μL of the donor bacteria and the same amount of the acceptor bacteria was mixed to perform the conjugation experiment. Fourteen positive clones were randomly selected, and PCR identification of GLO1 deficiency was performed using its outer primers. After successful identification, 500 μL of fresh culture broth of *Escherichia coli*-11/Δglo::Cm, was mixed with an equal volume of 50% sterile glycerol to store this strain at −80°C.

GLO1 Overexpression Plasmid Construction

To construct a GLO1 overexpression plasmid, the pUC19-EGFP vector (Miaolingbio. Inc, Wuhan, China) was used in this study. Firstly, the GLO1 fragment was synthesized from the entire gene, and the tool vector (ie, pUC19-EGFP vector) was digested with BamH1 (Takara Bio Inc., Kusatsu, Shiga, Japan) and Kpn1 (ThermoFisher, Waltham, Massachusetts, United States). Then the target DNA fragment was ligated to the restriction digest vector, and the recombination reaction was carried out at 4°C for 16 h. 10 μL of the reaction solution was taken out, transferred to TOP10 competent cells (Sangon Biotech, Shanghai, China) at 42°C for 45 sec, and then incubated in LB medium at 37°C for 16 h, which is resistant to ampicillin. The single clones grown on the plate are selected for sequencing. The sequencing results of positive clones are relatively correct. Finally, bacterial solution was saved and plasmid was extracted.

Table 1 RT-PCR Primer Sequences

Gene	Forward (5'-3')	Reverse (5'-3')
Add	CAACATTCGTCCCCAGACCA	TGACGGGCTGCATCTTCAAT
DeoD	TTATCGGTATGGGTGCCTGC	TTCCATTTCCACGCCGAGAA
GuaD	GATTTAGAGTACGCCCGCGA	GACCATTTTTGTGCCAGCGT
ApaH	ATGTAGAAGCGGTGCTGTCG	TCTTCCGGCGATTCTTTGCT
Adk	GCGTATCATTCTGCTTGGCG	CGCTTCTTTCATCGCGTCTG
PrdA	TAAAGAGAGGCCCGGAGACA	GGAAGAGCCAAACTCACCGA
SpeG	ACAGTGTTAAGCTACGCCCG	GGCTTTTTCGCCGTCACATT
Hdac10	GACCCCAGCGTCCTTTACTT	TTTGCCCCTCAAAGGCCAGT
Prodh	ATTGTGGGCTATGTGCGTGA	GAGAAAATCGACCGCTTCGC
CodA	GGCTACGGGCAGATTAACGA	GTGTGCTGGCAATCACCTTG
HchA	GCCGACTGATAACGGCAAAC	TCGCTGCATATTCGCTGTCT
FrdA	TCCTGATGACCGAAGGTTGC	GATAAACCACATCGCCACGC
GLO1	CTGGCGTTTGTTGGTTACGG	TGCGATAACCGTAGTACCGC
PpnK	CCTGGGTTTCCTGACTGACC	AGCCTGTTGGCGTCGAAATA
IlvE	ATATCTCTGAAGGCGCAGGC	AGGCTTGCTGAATGCGTTTG
Ppcdc	CAAGAACGCCCAGGATACGA	TTCGGGTTTCATGGCTCACA
VNN1	TCGAGACGGAGTTGACCTTG	AATCGGTTACACCCCACTCG
Tdk	CTTTGGTGCCGGGAAAGTCA	TTGTCGGACCATGCCAGTAA
DeoA	TGGACGTGAAAGTGGGTAGC	AAACAGACGCGGGTTACGAT
Tmk	CCGCGACATGGTTTTCACTC	ATCCCCGAGAACAGCATCAC
16S	TGCCTGATGGAGGGGGATAA	CCAGTGTTGCTGGTCATCCT

Determination of Bacterial and Supernatant β-Lactamase Content by ELISA

Bacterial and supernatant samples were collected from different groups of bacteria after 24 h culture. Double antibody sandwich method was used to determine the levels of different types of β-lactamases in cultured bacteria and supernatant, respectively, and the detailed procedures were performed according to the protocol of producer (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). In brief, the primary monoclonal antibody is coated on the microtiter plate to make a solid-phase antibody. The diluted samples containing different concentrations of β-lactamases were sequentially added, and then combined with the HRP-labeled secondary antibody to form an antibody-antigen-enzyme-antibody complex. After thorough washing, substrate TMB was added to develop color to blue, and acid was added to change color to yellow under the action of acid. The intensity of the color is positively correlated with the concentration of β-lactamase in the sample. The absorbance at a wavelength of 450 nm (OD_{450} value) was measured by a microplate reader, and the concentrations of β-lactamases in samples was calculated as a reference of the standard curve. Ten kinds of kit were utilized to detect concentrations of 10 types of β-lactamases, including BES type, CTX-M1 group, CTX-M2 group, OXA1 group, OXA2 group, OXA10 group, PER type, SHV type, TEM type, and VEB type.

Statistical Analysis

Statistical analysis was performed capitalizing on GraphPad Prism version 8 software. Data are represented as means ± standard deviation (SD) from more than three independent experiments. After testing for normality, data were analyzed by one-way ANOVA with multiple comparison test. A p-value of <0.05 was considered statistically significant. In this study, statistically significant differences are indicated by asterisks: * $P < 0.05$.

Results

Successful Isolation and Identification of Different Kinds of Clinical *Escherichia coli* Specimens

An antibiotic combination of ceftazidime, cefotaxime sodium and clavulanate potassium was added to MacConkey's medium to screen drug-resistant ESBLs-EC. The growth of standard bacteria, susceptible *Escherichia coli*, and drug-resistant ESBLs-EC in the medium is shown in Figure 1. The representative images showed that susceptible *Escherichia coli*, ie, No. 0 strain, did not grow in the medium (Figure 1A), the standard bacteria ATCC25922 did not grow either (Figure 1B), by contrast ESBLs-EC (No. 11 strain) formed positive several colonies which are visible by eye (Figure 1A). It confirms that we manage to isolate and culture ESBLs-EC from patient specimens. Furthermore, the largest colony from the No. 11 strain was selected for follow-up research.

Sequencing Results of the Whole Genome of Drug-Resistant Bacteria

Bacteria DNA was extracted from the largest colony of No. 11 strain, and the DNA library was successfully constructed and the whole genome map was analyzed to primarily identify DEGs. The quality of the data after quality control is good and meets requirements of the sequencing.

First of all, GO classification was performed on the obtained genes, and the GO terms of these genes were counted in the three categories. GO annotation demonstrated that 22 DEGs were grouped into functional subcategory of "biological process", 13 DEGs for "cellular component", and 14 DEGs for "molecular function", respectively (Figure 2A). Afterwards, the KEGG pathway enrichment analysis identified 3409 DEGs in five major groups: "cellular processes", "environmental information processing", "genetic information processing", "metabolism" and "organismal systems". The top five enriched biochemical pathways were "carbohydrate metabolism", "overview", "membrane transport", "amino acid metabolism" and "signal transduction" (Figure 2B). CAZy classification results displayed 174 DEGs in six major groups: "glycoside hydrolases", "glycosyl transferases", "carbohydrate esterases", "auxiliary activities", "carbohydrate-binding modules" and "polysaccharide lyase" (Figure 2C). Most DEGs were enriched in the group of glycoside

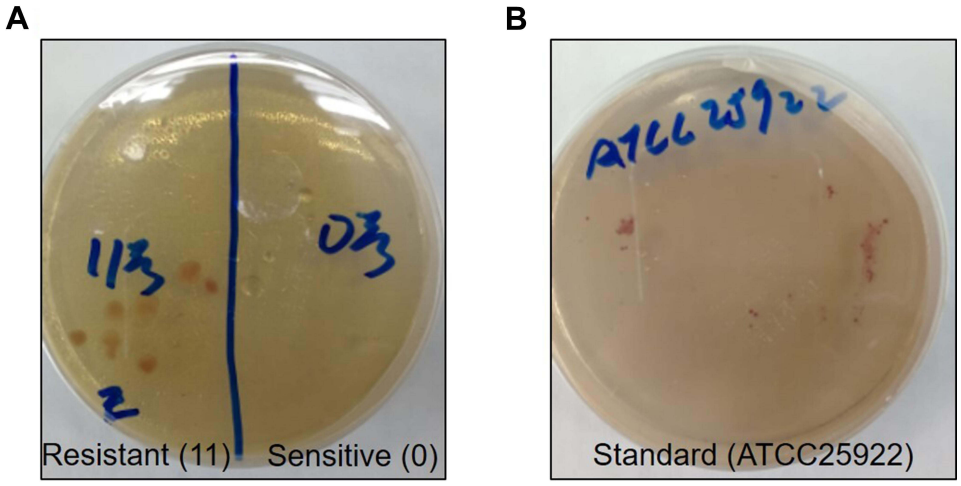

Figure 1 The growth of standard bacteria, susceptible and drug-resistant *Escherichia coli* in the culture medium.
Notes: (**A**) Right channel: No. 0 strain is a control susceptible *Escherichia coli* strain; left channel: No. 11 strain is a drug-resistant ESBLs-EC strain, showing several red round masses. (**B**) ATCC25922 is the control standard strain.

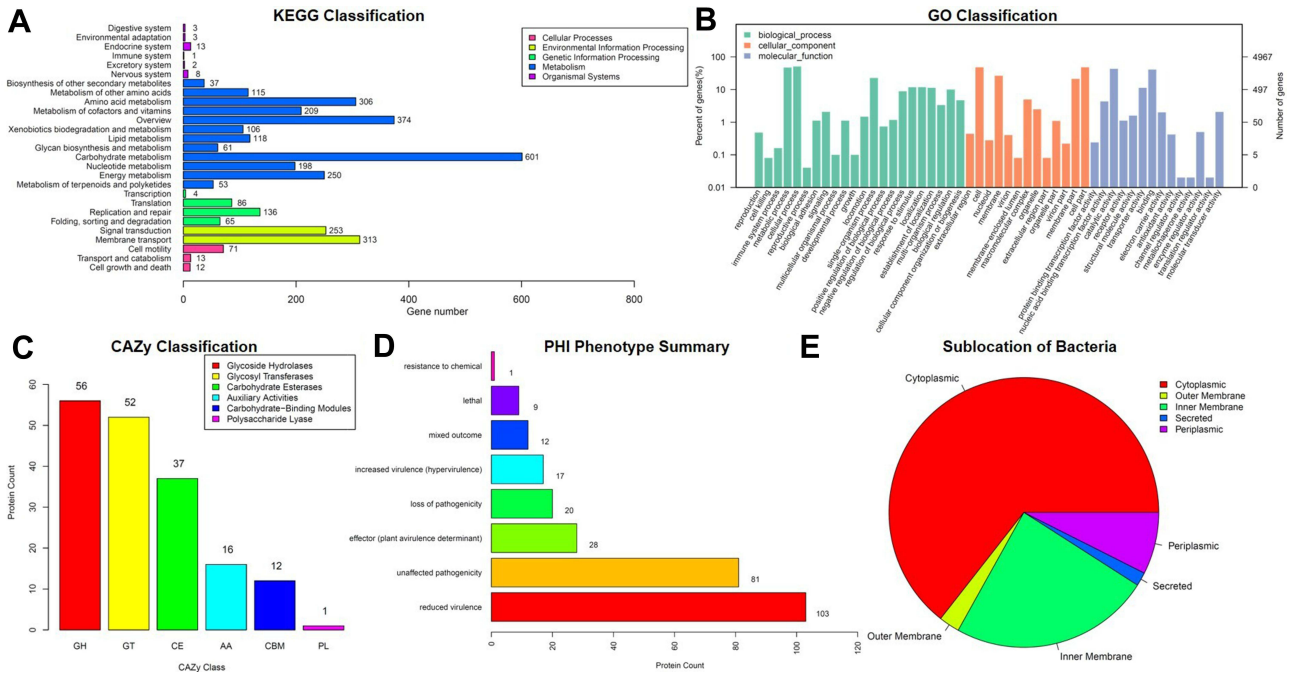

Figure 2 Sequencing results of the whole genome map of drug-resistant bacteria.

Notes: (**A**) Histogram of GO annotation distribution. These genes belong to each of three categories of Biological Process, Cellular Component, and Molecular Function term. The horizontal axis is the secondary classification of GO, and the vertical axis is the percentage of genes (left). Different colors represent different orthologs. (**B**) Histogram of KEGG pathway classification. The vertical axis is the name of the involved metabolic pathway, and the X axis is the number of genes annotated to the pathway. (**C**) The statistical histogram of CAZy functional classification. The horizontal axis is functional classification, and the vertical axis is the number of sequences. (**D**) Bar graph of pathogen–host interaction based on PHI phenotype summary. The vertical axis represents 9 different phenotypes of pathogen–host interaction, and the vertical axis is the number of genes. (**E**) Pie graph of prediction results of protein subcellular localization.

hydrolases. Analysis of bacterial pathogen host interactions through the PHI database demonstrated that most DEGs were associated with reduced virulence (Figure 2D). Lastly, overview of protein subcellular localization displayed that around 2410 DEGs were located in cytoplasm and 899 DEGs were in the inner membrane (Figure 2E). Taken together, the whole-genome sequencing data decipher the potential of DEGs involved in drug-resistant ESBLs-BL, and further offer some promising candidates.

Real-Time PCR Quantitative Results of Candidate Genes

Through the sequencing analysis of the whole genome of the No. 11 resistant ESBLs-EC, combined with the 18 differential pathways obtained from the previous association analysis,[16] we mainly screened the nodes where the protein and metabolites have changed in the meanwhile. In order to determine gene expression levels, we further performed RT-PCR. Twenty candidate genes were selected, including adenosine deaminase (add), purine nucleoside phosphorylase (deoD), guanine deaminase (guaD), nucleoside tetraphosphatase (apaH), adenylate kinase (adk), D-proline reductase proprotein (prdA), diamine N-acetyltransferase (speG), acetylputrescine deacetylase (Hdac10), proline dehydrogenase (Prodh), creatinine/cytosine deaminase (codA), D-lactate dehydratase/protein deglycase (hchA), fumarate reductase flavoprotein subunit (frdA), lactoylglutathione lyase/glyoxalase 1 (GLO1), nicotinamide adenine dinucleotide kinase (ppnK), branched-chain amino acid aminotransferase (ilvE), pantothenoylcysteine decarboxylase (Ppcdc), pantetheine Hydrolase (VNN1), thymidine kinase (tdk), thymidine Phosphorylase (deoA), and thymidylate kinase (tmk), as listed in Table 1. The mRNA expression of candidate genes in the standard bacteria ATCC25922, No. 0 susceptible *Escherichia coli* and No. 11 resistant ESBLs-EC was analyzed by real-time fluorescent quantitative PCR. The results showed that the mRNA levels of these genes did not change in susceptible *Escherichia coli* compared with the control bacteria ATCC25922. Of special note, the expression of speG, acetylputrescine deacetylase, GLO1 and pantothenoylcysteine decarboxylase was significantly upregulated in the drug-resistant ESBLs-EC, whereas the relative expression of other genes was downregulated (Figure 3), suggesting that these four genes may contribute to the drug-resistant property of ESBLs-EC.

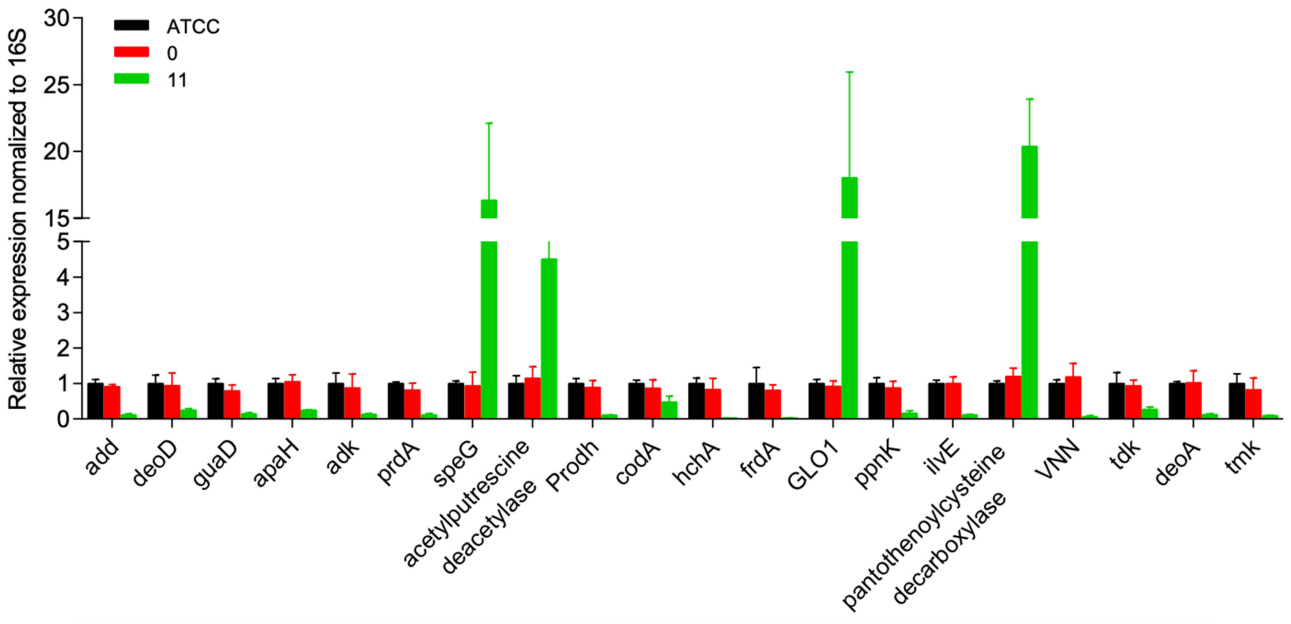

Figure 3 Relative gene expression of different types of bacteria (using 16s as the internal reference).
Note: ATCC is the standard bacteria, No. 0 strain is susceptible *Escherichia coli*, and No. 11 is ESBLs-EC.

Construction of Gene Knockout and Overexpression Strains

Based on the above results, we selected the GLO1 gene for verification. First of all, we knocked out the GLO1 gene by suicide plasmid method to construct an E. coli colony with low GLO1 expression, and overexpressed GLO1 by constructing a pUC19-EGFP vector with overexpression of GLO1. The results showed that the length of PCR amplified products of all 14 clones changed, which was in line with expectations. The original strain (ie, upstream homologous recombination arm-Cm resistance gene sequence-downstream homologous recombination arm) had an amplified product length of 1827 bp as shown in Figure 4A, and the newly-constructed strain had an amplified length of 2449 bp as shown in Figure 4B, implying that all fourteen clones could be positive clones. On the other hand, the GLO1 overexpression plasmid was successfully constructed and GLO1-overexpressed bacteria was correspondingly produced for next experiment, as shown in Figure 4C and D.

Supernatant Protein Level Determination of β-Lactamases by ELISA

Given the fact that there are few studies about the function of GLO1 in microorganisms and the effect of GLO1 expression changes on the drug-resistance of *Escherichia coli* is still unclear, we next investigated the correlation between GLO1 levels and β-lactamase production in supernatant and bacteria. To this end, we constructed GLO1 overexpression and knockout strains, and the effects of GLO1 gene changes on different subtypes of β-lactamases were analyzed through the ELISA kit. A total of 10 subtypes of β-lactamases were analyzed, including BES type, CTX-M1 type, CTX-M2 type, OXA1 type, OXA2 type, OXA10 type, PER type, SHV type, TEM type and VEB type. The results showed that changes in GLO1 gene expression level did not affect the expression of CTX-type of β-lactamase in bacteria (Figure 5A), but upregulated PER-type β-lactamase indeed (Figure 5B). Unfortunately, GLO1 knockout or overexpression had no significant effect on other types of β-lactamase either, such as TEM type, BES type, OAX type, SHV type and VEB type (Figure 5C–G). If we take a close look at PER type β-lactamase levels, we could find that the overexpression of GLO1 in standard bacteria ATCC25922 and *Escherichia coli* showed a significant increase in PER type β-lactamase, by contrast the PER type of β-lactamase of No. 11 strain with GLO1 knockout was significantly reduced. Furthermore, this reduction can be reversed by the transfection with GLO1 overexpression plasmid. These results together indicate that the change of GLO1 gene expression level has a significant correlation with the expression of PER type β-lactamase in *Escherichia coli* per se.

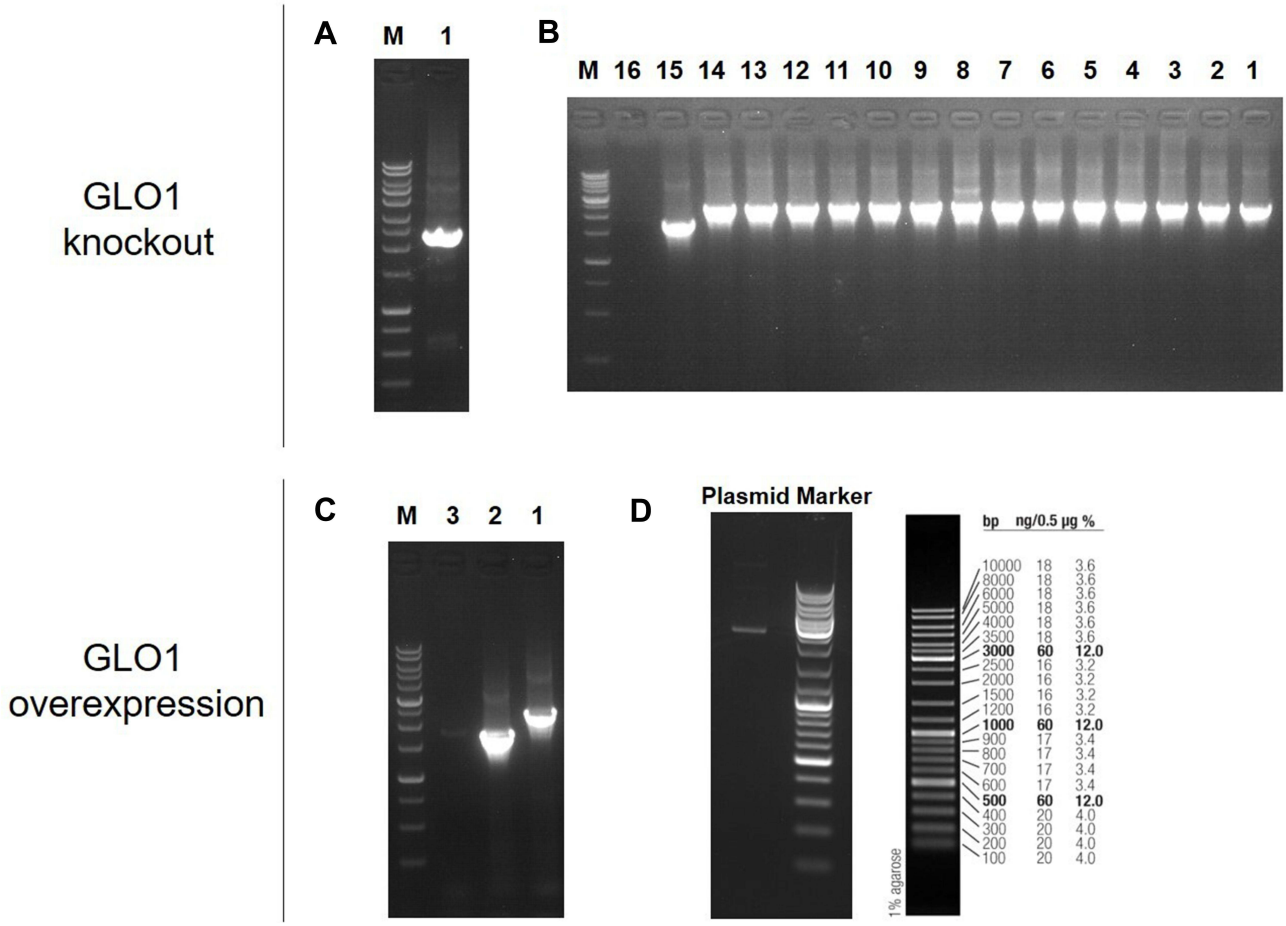

Figure 4 Verification of *Glo1* gene knockout and overexpression in bacteria strain.
Notes: (**A**) Construction of targeting fragments by fusion PCR technology (M: DNA molecular weight standard. The molecular weights from top to bottom are: 10,000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600, 400, 200 bp, among which 1000bp is highlighted). The length of targeting sequence is 2291bp. (**B**) PCR identification of outer primers of GLO1 knockout flora gene, and the same DNA molecular weight standard was used. No. 1–14: Amplification results of the outer primers of clone 1–14; No. 15: amplification result of original strain; No. 16: amplification result of negative control without template. (**C**) Monoclonal bacterial solution inoculated with cloning bacterial solution No. 1 was verified with outer primers. Lane 1: the amplification result of the outer primer of the bacterial solution; lane 2: the amplification result of the original strain; lane 3: the amplification result of the negative control without template. (**D**) DNA results of GLO1 overexpression plasmid (expected fragment 417+3.4K).

After the correlation between GLO1 expression and β-lactamase production was overserved in bacteria, we are curious about whether the secretion of GLO1 changes in the same experiments. Then, similar results were observed in the supernatant of different groups of bacteria (Figure 6). Taken together, these results indicate that the change of GLO1 gene expression level has a significant correlation with the expression of PER type β-lactamase in *Escherichia coli*.

Discussion

Since *Escherichia coli* can result in severe and even fatal infections in both human beings and animal, it occupies an important position in the microbiological world. Of major concern is that *Escherichia coli* has a strong ability to accumulate drug resistance genes, in most cases through horizontal gene transfer, although this strain initially can be sensitive to the majority of clinically applied antibiotics.[17,18] So far, the most common resistance genes of *Escherichia coli* mainly include various well-known genes coding ESBLs, carbapenemases, 16S rRNA methylase such as armA in *Acinetobacter baumannii*, plasmid-mediated quinolone resistance (PMQR) genes, and mobilized colistin resistance (MCR) genes.[19–22] Admittedly, the identification of these resistant genes and relevant targeting antibiotic development effectively solve plenty of AMR clinical issues to some extent. However, current situation is still challenging due to the undefined puzzles in the context of resistant gene exploration. Among these genes, ESBL attracts quite a lot of attentions

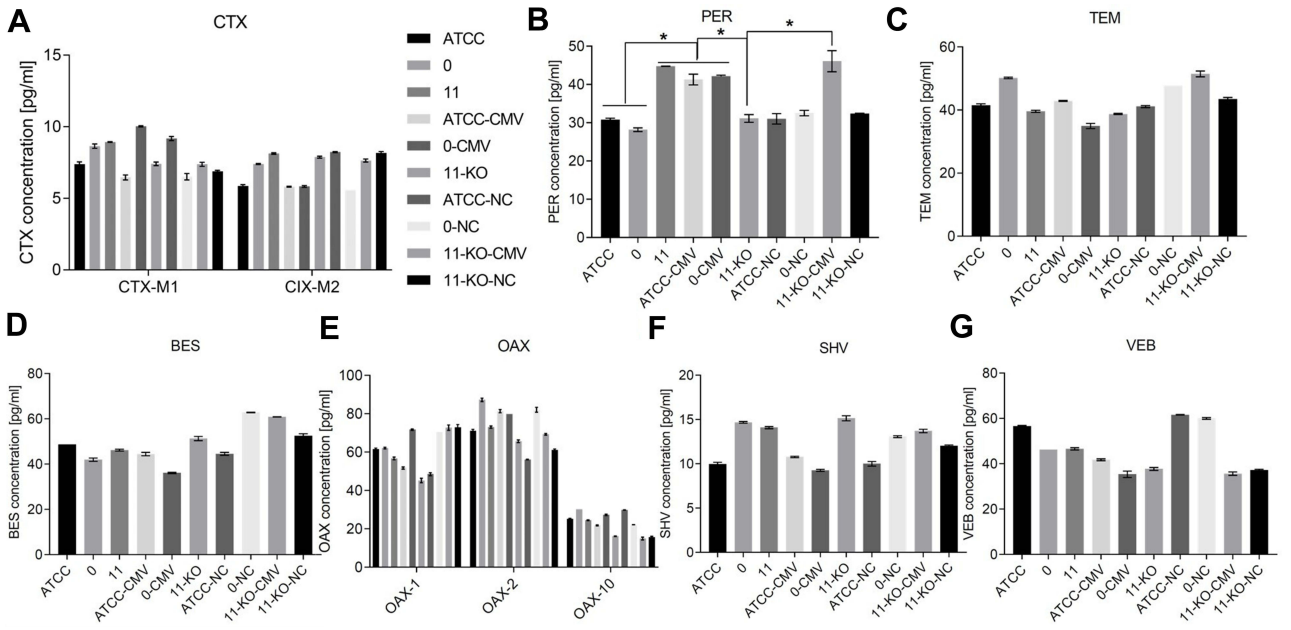

Figure 5 Determination of β-lactamase content in bacteria by ELISA.

Notes: (**A**) Detection of CTX type of β-lactamase. (**B**) Detection of PER type of β-lactamase. (**C**) Detection of TEM type of β-lactamase. (**D**) Detection of BES type of β-lactamase. (**E**) Detection of OAX type of β-lactamase. (**F**) Detection of SHV type of β-lactamase. (**G**) Detection of VEB type of β-lactamase. 11-KO: ESBLs-EC with knockout of GLO1; 11-KO-CMV: GLO1-deficient ESBLs-EC with transfection of GLO1-overexpressing plasmid; 11-KO-NC: GLO1-deficient ESBLs-EC transfected with blank overexpression plasmid; ATCC-CMV: standard bacteria transfected with blank overexpression plasmid; ATCC-NC: standard bacteria with GLO1 overexpression; 0-CMV: susceptible *Escherichia coli* with GLO1 overexpression; 0-NC: blank overexpression control bacteria. *$P < 0.05$.

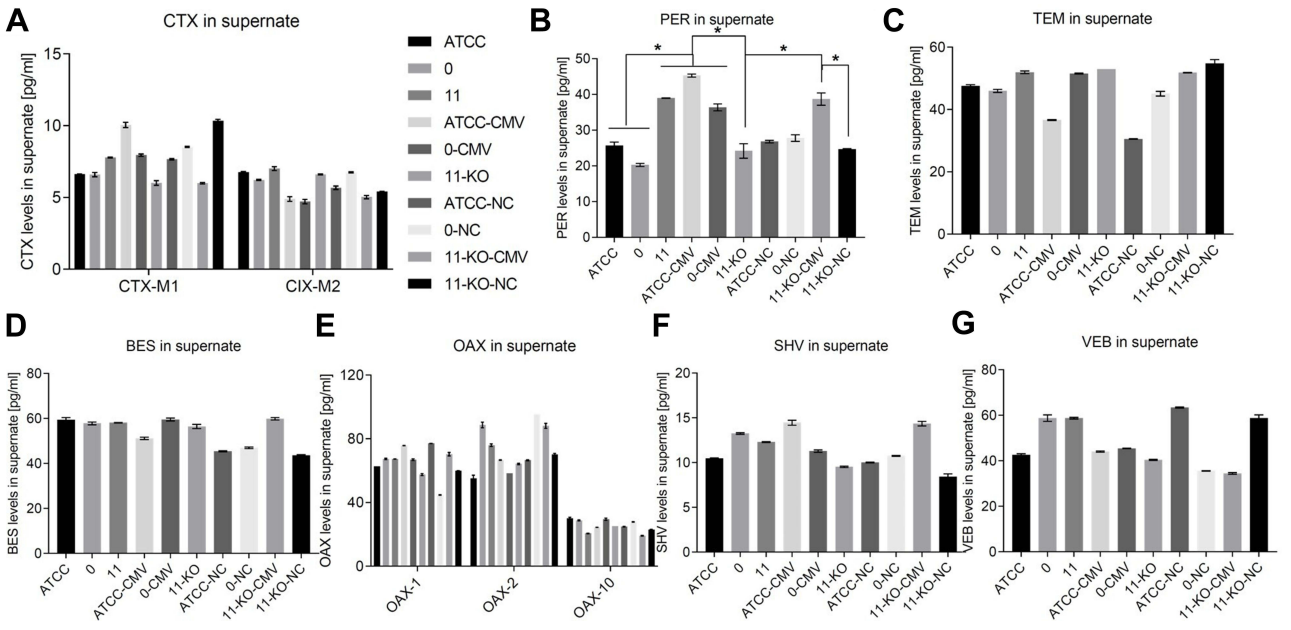

Figure 6 Determination of β-lactamase content in supernatant by ELISA.

Notes: (**A**) Detection of CTX type of β-lactamase. (**B**) Detection of PER type of β-lactamase. (**C**) Detection of TEM type of β-lactamase. (**D**) Detection of BES type of β-lactamase. (**E**) Detection of OAX type of β-lactamase. (**F**) Detection of SHV type of β-lactamase. (**G**) Detection of VEB type of β-lactamase. *$P < 0.05$.

as ESBLs-producing *Escherichia coli* is easily induced by antimicrobial agents, which further serve as the main indicators to evaluate estimate antimicrobial resistance burden.[23,24] In hindsight, it was previously believed that ESBLs-EC are usually resistant to ceftazidime and cefotaxime and other cephalosporins, however display higher sensitivity to β-lactamase inhibitors such as clarithromycin, sulbactam or tazobactam.[25] Even so, the extensive

application of β-lactamase inhibitors is not enough to treat or improve this kind of clinical condition, also indicating that there may exist other molecular mechanisms involved in the development of ESBLs-EC resistance. Therefore, this is the major reason why this study focuses on ESBLs-producing *Escherichia coli* and performs deeper investigation based on current knowledge.

Following the indications from literatures and our preliminary study, we have two directions. One is to identify other pivotal resistant genes directly or indirectly associated with the production of ESBLs in *Escherichia coli*, and the other is to target at specific type of β-lactamase. To this end, our research group previously combined proteomics with metabolomics analysis of clinical specimens, and further revealed some differently expressed genes, proteins, pathways as well as metabolites in the antibiotic resistance of ESBLs-EC, providing solid evidence for mechanism study.[16] In addition to proteomics and metabolomics as potent tools in the research of bacterial drug-resistance,[26–29] we applied whole-genome sequencing (WGS) on same clinical samples in this study to firstly identify DEGs. Based on this, we further performed RT-PCR to determine their relative expression levels. Surprisingly, we found that the levels of these four genes including speG, acetylputrescine deacetylase, GLO1 and pantothenoylcysteine decarboxylase were significantly increased in ESBLs-EC in comparison with susceptible strains, whereas the expression of other genes was decreased. Subsequent experiments utilizing gene knockout and overexpression strains confirmed that GLO1 gene expression was positively correlated with the production of PER type of β-lactamase. Of special note, the GLO1 gene expression level only affected the production of PER type of β-lactamase, but had no significant effects on other types of β-lactamases. However, there is still a lack of mechanistic studies about the association between GLO1 and PER type of β-lactamase so far. Based on our findings, we could make a speculation that GLO1 may act as a specific regulator for PER type of β-lactamase production, which still needs more powerful experimental evidence to confirm further. Even so, these findings together suggest that GLO1 may contribute to the drug-resistance of *Escherichia coli* through inducing PER type of ESBLs, offering novel target for new antibiotic development in the near future. As reviewed by Panter et al recently, more and more proteomics, genomics and metabolomics studies in this field are urgently encouraged to unravel other potential mechanisms for antibiotic-resistance.[30]

Based on our findings, different types of ESBLs will be briefly discussed here. In this study, 10 subtypes of ESBLs were detected, including BES type, CTX-M1 type, CTX-M2 type, OXA1 type, OXA2 type, OXA10 type, PER type, SHV type, TEM type and VEB type, which basically covered the most common types of ESBLs reported.[31–33] Among them, SHV- and TEM-ESBLs were initially studied at the end of the twentieth century, and acted as the dominant form afterwards.[34,35] From the beginning of 21st century, CTX-M-ESBLs were newly identified and then extensively studied.[36] Other subtypes of ESBLs were gradually discovered and under really active investigation in the past two decades. In brief, CTM-M-, SHV- and TEM-ESBLs are still predominant resistant genes for *Escherichia coli* with a wide range of variant genes. But there emerge other subtypes of ESBLs with the popular application of antibiotics, for example PER- ESBLs, which possesses the reverse Ω loop and the extended B3-β chain and is a new member of the ESBL family.[37] Therefore, current research should pay more attention to these less studied ESBLs. Our experiment also checked these three genes, however did not observe any significant changes, which is in line with this view.

Given the finding that GLO1 levels are positively correlated with the production of PER-ESBLs, the function of GLO1 in *Escherichia coli* infection and possible associations with PER-ESBLs were further investigated. Therefore, it will also be discussed here. The original function of GLO1 is to catalyze the condensation of methylglyoxal.[38] Although there are some studies reporting the involvement of GLO1 in bacteria, yeast, and protozoa, etc., no papers mentioned the potential of GLO1 in the AMR of *Escherichia coli*. So, this finding in our study definitely needs more study to confirm further. Remarkably, with the generation of GLO1 deficient mice, it is available to verify this association in vivo.[39] However, the identification of other bacterial GLO1 mutation might make this problem more complicated.[40] In addition to GLO1, we also found that speG displayed upregulation in ESBLs-EC compared to susceptible *Escherichia coli*, which is at least partially consistent with the results from recent two studies.[41,42] In the future, we will continue with the study with other three upregulated genes.

Conclusion

In conclusion, we unexpectedly discovered that the high expression of GLO1 directly affects the production of PER subtype of β-lactamase in *Escherichia coli* in this study, the subsequent successful construction of GLO1 overexpression and knockout bacterial strains strongly verified this finding furthermore. Of course, we acknowledged that this study still has some limitations and needs to be explored more deeply. In the future, it should be clarified or confirmed that the combinative use of GLO1 inhibitors with β-lactamase inhibitors could achieve stronger clinical efficacy, providing a certain experimental and theoretical basis for the future design of specific targeted GLO1-related β-lactamase inhibitors, in order to solve such clinical problems of drug resistance effectively.

Ethical Statement

The study was conducted in accordance with the Declaration of Helsinki. Anonymized clinical samples obtained during routine hospital procedures were used for this study.

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Author Contributions

All authors made significant contributions to conception, study design, execution, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; have agreed on the journal submitted; and agree to be accountable for all aspects of the work.

Disclosure

All the authors have no competing interests to declare.

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