

# iTRAQ-Based Proteomics Reveals Potential Anti-Virulence Targets for ESBL-Producing *Klebsiella pneumoniae*

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**Purpose:** Treatment of infections with *Klebsiella pneumoniae* strains producing extended-spectrum beta-lactamases (ESBLs) is challenging due to the coexistence of multiple resistance mechanisms and the hypervirulent variant. Therefore, new targets or more effective treatment options aimed at ESBL-producing *Klebsiella pneumoniae* are urgently needed.

**Materials and Methods:** Here, we collected ESBL-producing and non-ESBL *Klebsiella pneumoniae* isolates and studied their differences from a proteomic point of view.

**Results:** We revealed *treA*, *wza*, *gnd*, *rmlA*, *rmlC*, *rmlD*, *galE*, *aceE*, and *sucD* as important virulence-related proteins in ESBL-producing *Klebsiella pneumoniae*, distinct from those in non-ESBL strains.

**Conclusion:** Our findings provide plausible anti-virulence targets and suggest new therapeutic avenues against ESBL-producing *Klebsiella pneumoniae*.

**Keywords:** *Klebsiella pneumoniae*, extended-spectrum beta-lactamases, proteomics, anti-virulence

## Introduction

Antibiotics can kill or inhibit growth of bacteria by disrupting biosynthetic processes or essential cellular pathways. However, the misuse of antibiotics has led to the evolution of antimicrobial resistance (AMR) genes and the selection of multiple antibiotic-resistant bacteria. This has prompted the development of alternative strategies to treat drug-resistant bacterial infections.<sup>1</sup> When bacteria invade the host, they activate virulence factors including toxins, adhesins, capsular polysaccharides (CPS), lipopolysaccharide (LPS), and exopolysaccharide (EPS). The activated virulence factors may aid bacterial attachment to the host cells, cloak the subcapsular epitopes on the surface, and escape the host immune response.<sup>2,3</sup> Therefore, preventing the expression or activation of bacterial virulence factors could be an alternative strategy, distinct from the classic antibiotic mechanisms, which will not interfere with bacterial survival, allowing the host to fight the infection through immune responses.

Infections due to Gram-negative bacteria are a cause of morbidity and mortality worldwide. Extended-spectrum beta-lactamases (ESBLs) render treatment of these pathogens particularly difficult due to the limited options of effective antibiotics.<sup>4</sup> Due to their virulence factors, ESBL-producing *Klebsiella pneumoniae* strains are significantly more resistant to the antibacterial activity of serum than non-ESBL-producing *K. pneumoniae* strains.<sup>5,6</sup> However, the potential additional factors that

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render ESBL-producing *Klebsiella pneumoniae* more virulent remain elusive. In this study, we collected ESBL-producing and non-ESBL-producing *K. pneumoniae* isolates randomly, and studied their proteomic differences. We identified important virulence-related proteins in ESBL-producing *K. pneumoniae* distinct from those in the non-ESBL strains. Furthermore, we report novel plausible anti-virulence targets and promising treatment options against infection with ESBL-producing *K. pneumoniae*.

## Materials and Methods

Isobaric tags for relative and absolute quantification (iTRAQ) Reagent-4/8plex Multiplex Kit (AB Sciex). Antibodies: anti-GALE (Proteintech, 14,414-1-AP), anti-Trehalase (Abcam, 133,808), anti-PGD (Proteintech, 14,718-1-AP), anti- $\beta$ -actin (ABclonal, AC004), HRP Goat Anti-Mouse IgG (ABclonal, AS003HRP), HRP-conjugated Affinipure Goat Anti-Rabbit IgG (Proteintech, SA00001-2). Chemiluminescent HRP substrate (Millipore, WBKLS0100).

## Methods

### Sample Collection

ESBL-producing *K. pneumoniae* (n=60) and sensitive *K. pneumoniae* (n=60) were collected by the microbiology lab at the Second Hospital of Jilin University. All the selected ESBL-producing isolates carried resistance genes CTX-M, SHV and TEM simultaneously. In addition, ensure that the drug sensitivity results of all ESBL-producing or sensitive isolates are the same. For iTRAQ labeling experiments, 30 ESBL-producing isolates and 30 non-ESBL-producing *K. pneumoniae* isolates were randomly distributed in six groups. For validation experiments, another 30 isolates from each type were employed. The colonies were incubated in 200 mL LB broth liquid medium (Invitrogen, 10,855,001) and cultured for 16–18 h at 37°C in shaking conical flasks. Thereafter, the medium was centrifuged at 3500 g for 15 min, and the sediment was collected. Subsequently, the sediments were washed three times with sterile PBS.

### Protein Extraction and Quantification

For both the iTRAQ labeling experiment and the validation experiment, every 10 isolates of sediments from each type were mixed as one group. Each group of sediments were all added to the SDT pyrolysis solution (4% SDS, 100 mM Tris-HCl, pH 7.6), transferred to lysing matrix A tubes, and broken by MP homogenizer (24 × 2, 6.0 M/s, 60 s, twice). Subsequently, after ultrasonication, the homogenate was

boiled for 10 min, followed by centrifugation at 14,000 g for 15 min. The supernatants were then filtered using 0.22  $\mu$ m filters and collected. Proteins were quantified using BCA (Beyotime, P0012) method. The samples were stored at –80°C.

### SDS-PAGE Electrophoresis

For the iTRAQ labeling experiment, 20  $\mu$ g protein from each sample was mixed with 6X loading buffer solution and boiled for 5 min. Proteins were separated using 12% SDS-PAGE electrophoresis (constant 250 V, 40 min), followed by Coomassie Brilliant blue staining. For Western blot, 50  $\mu$ g protein from each sample was separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were blocked by Tris-buffered saline solution containing 5% non-fat milk before incubation with primary and secondary HRP-conjugated antibodies, prepared in blocking buffer. Bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Pittsburgh, PA, USA).

### Protein Digestion and iTRAQ Labeling

Prior to the iTRAQ labeling experiments, equal quality of protein from each group extracted as described above were trypsinized according to the FASP protocol.<sup>7</sup> The eluted peptides were quantified using a NanoDrop (Thermo Fisher scientific ND2000).

Subsequently, 100  $\mu$ g of peptides from each group were iTRAQ-labeled using an 8-plex iTRAQ labeling kit following the manufacturer's protocol. Three controls (non-ESBL-producing *K. pneumoniae*) were labelled with iTRAQ reagents 115, 116, and 117, and 3 EBSL<sup>+</sup> (ESBL-producing *K. pneumoniae*) groups were labelled with iTRAQ reagents 118, 119, and 121, respectively.

### High pH One-D Reverse Phase Chromatography Fractionation

Fractionation of the peptide mixture was performed on an Agilent 1260 Infinity II HPLC system. Buffer A contained 10 mM HCO<sub>2</sub>NH<sub>4</sub>, 5% ACN, pH 10.0; buffer B contained 10 mM HCO<sub>2</sub>NH<sub>4</sub>, 85% ACN, pH 10.0. The column was initially equilibrated in buffer A. The samples were separated on the chromatographic column at a flow rate of 1 mL/min. The gradient of liquid phase was the following: buffer B 0%, 0 min–25 min; buffer B 0%–7%, 25 min–30 min; buffer B 7%–40%, 30 min–65 min; buffer B 40%–100%, 65 min–70 min; buffer B 100%, 70 min–85 min. During elution, UV absorbance was monitored at 214 nm. Fractions were collected every minute during the course of

the run. Subsequently, all 36 fractions were collected, lyophilized and kept at  $-80^{\circ}\text{C}$  prior to mass spectrometry analysis.

### Mass Spectrometry Analysis

The collected fractions were examined by Easy nLC system. Buffer A contained 0.1% formic acid aqueous; buffer B contained 0.1% formic acid acetonitrile (acetonitrile concentration 80%). The column was initially equilibrated in 100% buffer A. The samples were separated on the column (Acclaim PepMap RSLC  $50\ \mu\text{m} \times 15\ \text{cm}$ , nano viper, P/N164943) at a flow rate of 300 nl/min. The gradient of liquid phase was the following: buffer B 6%, 0 min-5 min; buffer B 6%-28%, 5 min-45 min; buffer B 28%-38%, 45 min-50 min; buffer B 38%-100%, 50 min-55 min; buffer B 100%, 55 min-60 min.

After separation on the chromatography column, the samples were analyzed by Q Exactive mass spectrometer (Thermo Fisher Scientific). Key parameter settings for the Q Exactive mass spectrometer were: (1) First grade MS parameters: Resolution: 70,000, AGC target:  $3e6$ , Maximum IT: 50 ms, Scan range: 350 to 1800 m/z; (2) Second grade MS parameters: Resolution: 17,500, Maximum IT: 45 ms, Isolation window: 2 m/z, Microscans: 1, Maximum IT: 45ms, Normalized Collision Energy 30eV.

### Database Search for Protein Identification

The original map file produced by Q Exactive was transformed into. Mgf file by Proteome Discoverer 2.1 (Thermo Fisher Scientific) and submitted to the server MASCOT2.6 for database retrieval through the tools in the software. The database for the search was Uniprot\_KlebsiellaPneumoniae\_223866\_20180301 (<http://www.uniprot.org>). Then, the library file (.Dat) formed on the MASCOT server was transferred back to the software through Proteome Discoverer 2.1, and the data were filtered based on the standard of false discovery rate (FDR) $<0.01$ . Finally, highly reliable qualitative results were obtained.

In the process of quantitative analysis, the Proteome Discoverer 2.1 software was applied to extract the peak strength of the peptide ion. The search parameters were: (1) peak integration, integration window tolerance: 20 ppm; integration method: most confident centroid; (2) scan event filters, mass analyzer: FTMS; MS order: MS2; activation type: HCD; (3) quantification – general, peptides to use: unique; (4) normalization scaling, normalization mode: total peptide amount; scaling mode: on channels average.

All identified proteins and peptides are listed in [S1](#) and [S2](#) File.

### Bioinformatics Analysis

#### Analysis of Differentially Expressed Proteins

For computing the differential ratio, peptides and proteins having an ion score greater than 99% confidence were included. Peptides with low confidence score of 1%, without peaks corresponding to iTRAQ labels, as well as the peptides found in multiple proteins were excluded. The quantitative estimates for each protein included: protein abundance; average protein abundance of corresponding groups; differential expression fold change ratios; p-value; and FDR. After identification and quantification, proteins identified at 1% FDR with a confidence threshold of 95% and p-values  $<0.05$  were retained. The volcano plot of fold-change ratios was set up, and the proteins with fold change ratio  $>1.2$  and  $<0.833$  were screened out as up-regulated and downregulated proteins in  $\text{ESBL}^+$  groups compared with  $\text{ESBL}^-$  groups, respectively.

#### Enrichment Analysis of Differentially Expressed Proteins and Construction of Protein-Protein Interaction (PPI) Network

The enrichment analysis of GO annotations ([www.geneontology.org](http://www.geneontology.org)) was performed to evaluate the significance of a GO term enrichment. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (<http://www.genome.jp/kegg/>) was performed to annotate the functional pathways of all identified proteins or peptides. KEGG pathway enrichment analysis was similar to that of GO. The significance of protein enrichment in each pathway was calculated by Fisher accurate test (Fisher's Exact Test), taking KEGG pathway as unit and all qualitative proteins as background. The significantly affected metabolic and signal transduction pathways were determined. Certain differentially expressed proteins were mapped by the online Search Tool for the Retrieval of Interacting Genes (STRING) database (<https://version-10-5.string-db.org/>) to construct the PPI network and disclose the possible connections among proteins. The PPI network was constructed by setting the minimum required interaction score to medium confidence (0.4). The active interaction sources included were “experiments”, “textmining”, and “database”. The setting parameters for maximum number of interactors to show for first shell and second shell were respectively “none/query protein only” and “none”.

## Results

### Identification of Differentially Expressed Proteins

The Volcano plot generated by Protein pilot in function of the protein ratio obtained by comparison of ESBL<sup>+</sup> *K. pneumoniae* and non-ESBL *K. pneumoniae* groups is depicted in [Figure 1](#). To identify differentially expressed proteins, 4340 proteins were used as background to screen candidates with ratio >1.2 as upregulated proteins or <0.833 as downregulated proteins. The abundance ratio values of certain differentially expressed protein were similar in three independent experiments. After statistical analysis, in total 934 proteins were considered upregulated and 726 were downregulated in ESBL<sup>+</sup> compared to controls. The basic information of differentially expressed proteins, the abundance ratio values as well as the *p* values are listed in [S3](#) File.

### Functional Enrichment Analysis of Differentially Expressed Proteins

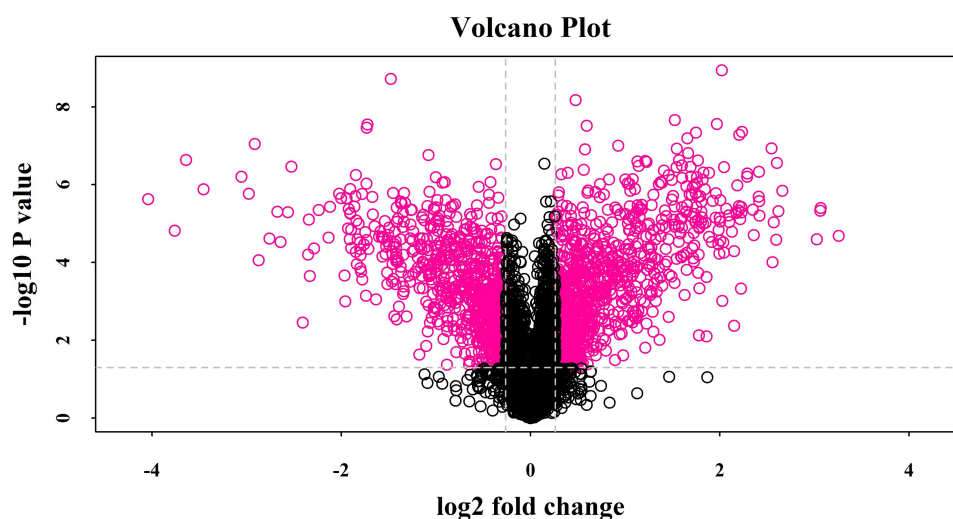
Functional enrichment analysis of the 1610 differentially expressed proteins identified 1138 proteins in the category of “biological process”, 767 in “molecular function”, and 144 in “cellular component”. The functional terms in each category, ranked by statistical significance, are summarized in [S4\(1–3\)](#) File. As presented in [Figure 2](#), the differentially expressed proteins were mostly enriched in “cellular process”, “metabolic process”, “single-organism process”, “catalytic activity”, and “binding”.

### Construction of Protein–Protein Interaction Network

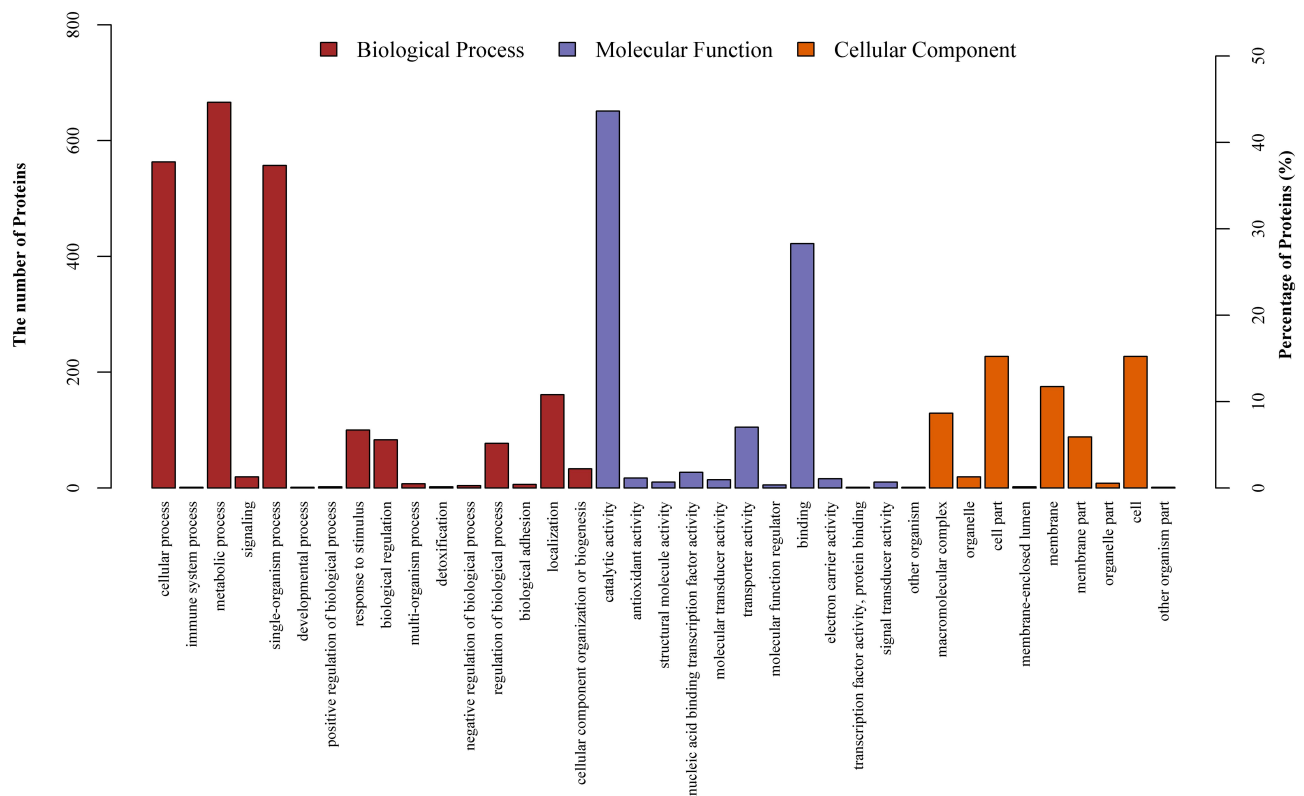
Subsequently, we further screened out the differentially expressed proteins with fold change ratio  $\geq 2$  and  $\leq 0.5$ . The 264 upregulated and 182 downregulated proteins were combined and mapped by online STRING database to disclose the possible connections among the 446 differentially expressed proteins and visualize the protein–protein interaction (PPI) network ([Figure 3](#)). The screened differentially expressed proteins formed a complex regulatory network containing 90 nodes and 115 edges, with an average node degree of 2.56 and a clustering coefficient of 0.421 ([Figure 3](#)). The number of expected edges was 71, and the PPI enrichment *p*-value was  $8.58e-07$ .

### The Potential Anti-Virulence Targets in ESBL-Producing *Klebsiella pneumoniae*

Among the screened differentially expressed proteins in ESBL<sup>+</sup> *K. pneumoniae*, and based on the center-nodes in the constructed network ([Figure 3](#)) and the ratios in [S3](#) File, we screened out the top 9 significantly different proteins that showed the potential to be anti-virulence targets in ESBL-producing *K. pneumoniae*. The top 9 candidates included periplasmic trehalase (treA), integral outer membrane lipoprotein (Wza), 6-phosphogluconate dehydrogenase (gnd), dTDP-L-rhamnose synthesis (*rmlA*, *rmlC* and *rmlD*), UDP-galactose-4-epimerase (galE), pyruvate dehydrogenase E1 component (aceE), and succinate-CoA synthetase subunit alpha (sucD) ([Table 1](#)).



**Figure 1** Volcano plot showing log<sub>2</sub> fold change plotted against -log<sub>10</sub> adjusted P value for ESBL<sup>+</sup> *Klebsiella pneumoniae* samples versus non-ESBL *Klebsiella pneumoniae* samples. Data points in the upper right (fold change > 1.2) and upper left (fold change < 0.83) sections with *P* < 0.05 represent proteins that are significantly different in ESBL<sup>+</sup> *Klebsiella pneumoniae* compared with non-ESBL *Klebsiella pneumoniae*.



**Figure 2** Gene Ontology (GO) classification of differentially expressed proteins by iTRAQ-based proteomics experiments between ESBLs<sup>+</sup> *Klebsiella pneumoniae* and non-ESBL *Klebsiella pneumoniae*. The differentially expressed proteins are grouped into three GO terms: biological process, cellular component, and molecular function. The y-axis indicates the number and percent of proteins in each GO term.

## Western Blot Analysis to Validate the Representative Differentially Expressed Proteins

Three proteins (*treA*, *gnd*, *galE*) were selected as representative biomarkers of ESBL-producing *K. pneumoniae*, and validated using Western blot to confirm the proteomic data. Consistently, ESBL-producing *K. pneumoniae* isolates showed higher expression levels of *treA*, *gnd*, and *galE* (Figure 4A–C).

## Discussion

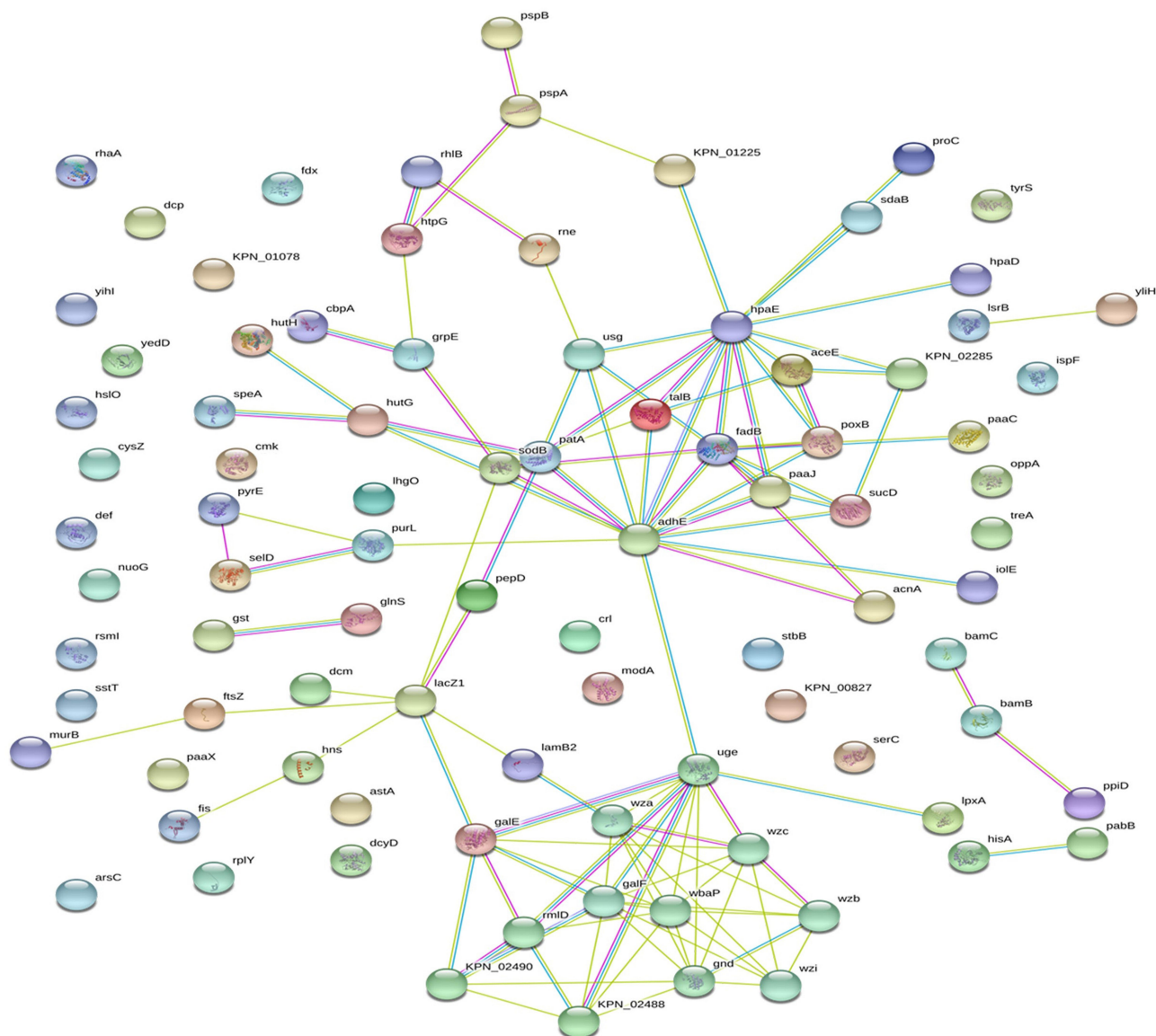
*K. pneumoniae* causes a wide range of infections in humans, including pneumonia, urinary tract infections, bacteremia, and liver abscesses. The alarming increase in the prevalence of drug-resistant *K. pneumoniae*, such as ESBL- or carbapenems-producing *K. pneumoniae*, has raised our awareness of the urgency of non-antibiotic based strategies against antibiotic-resistant infections.

Virulence is an important strategy for *K. pneumoniae* to grow in the tissues and protect itself from the host immune response. Four major classes of virulence factors, including

capsule, lipopolysaccharide, fimbriae, and siderophores, have been well characterized in *K. pneumoniae*.<sup>8</sup> Nevertheless, much is still unknown about additional virulence factors in *K. pneumoniae*. In this study, by performing comparative proteomics of clinically isolated ESBL-producing *K. pneumoniae* versus non-ESBL strains, we revealed 9 highly expressed virulence factors of ESBL-producing *K. pneumoniae*.

Trehalose is a disaccharide formed by two glucose molecules, and is widely distributed in nature in bacteria, fungi, plants, and invertebrates.<sup>9</sup> Trehalose not only serves as an energy storage molecule in bacteria, but also plays a role in the virulence of some microorganisms.<sup>10</sup> Mutations that abolish trehalose production in various fungi may reduce virulence.<sup>11</sup>

In the periplasm, trehalose can be degraded into two molecules of glucose by periplasmic trehalase (*treA*).<sup>10</sup> Vanaporn<sup>10</sup> showed that *treA* gene deletion in *B. pseudomallei* increases the tolerance to thermal stress and results in lower biofilm production than in the wild type. Moreover, mice challenged with *treA*-deleted *B. pseudomallei* survived until the end of their study, whereas mice challenged with wild type



**Figure 3** Differentially expressed proteins were combined to build a regulatory network by STRING. The differentially expressed proteins are represented as nodes, and the relationship between two nodes is represented as an edge. The empty nodes represent the proteins of unknown 3D structure, while the filled nodes indicate that some 3D structure is known or predicted. The interaction information between proteins from curated databases is represented by a blue edge; experimentally determined: purple edge; predicted interactions: cyan edge; and text mining: light green edge. The network contained 90 nodes and 115 edges with an average node degree of 2.56 and a clustering coefficient of 0.421. The PPI enrichment p-value was equal to  $8.58e-07$ .

*B. pseudomallei* succumbed to the infection. In our study, the ratio of *treA* between ESBL<sup>+</sup> and non-ESBL *K. pneumoniae* was the highest among all differentially expressed proteins. Therefore, we speculate that the over-expression of *treA* plays an important role in ESBL<sup>+</sup> *K. pneumoniae* virulence. To date, only a few studies have explored the role of *treA* in *K. pneumoniae*, especially in the antibiotic-resistant strains. Further studies are thus needed to reveal its suitability as anti-virulence target.

OmpK35 and OmpK36 are porins closely related to the antibiotic resistance in *K. pneumoniae*, including ESBL-

producing and carbapenem-resistant strains.<sup>12,13</sup> The porins can also act as virulence factors during *K. pneumoniae* infection.<sup>14</sup> Most drug-sensitive *K. pneumoniae* strains express both OmpK35 and OmpK36, whereas most ESBL<sup>+</sup> *K. pneumoniae* strains express only OmpK36.<sup>15</sup> Deletion of OmpK36 reduces the virulence of *K. pneumoniae* by increasing neutrophil-mediated phagocytosis in vivo. However, the deletion also leads to increased antimicrobial resistance, especially associated with decreased susceptibility to carbapenems.<sup>12,14,16,17</sup> In this study, OmpK36 ranked second (ratio=8.38) among the differentially expressed proteins,

**Table 1** Top 9 Proteins Potential to Be Anti-Virulence Targets in ESBL-Producing *Klebsiella pneumoniae*

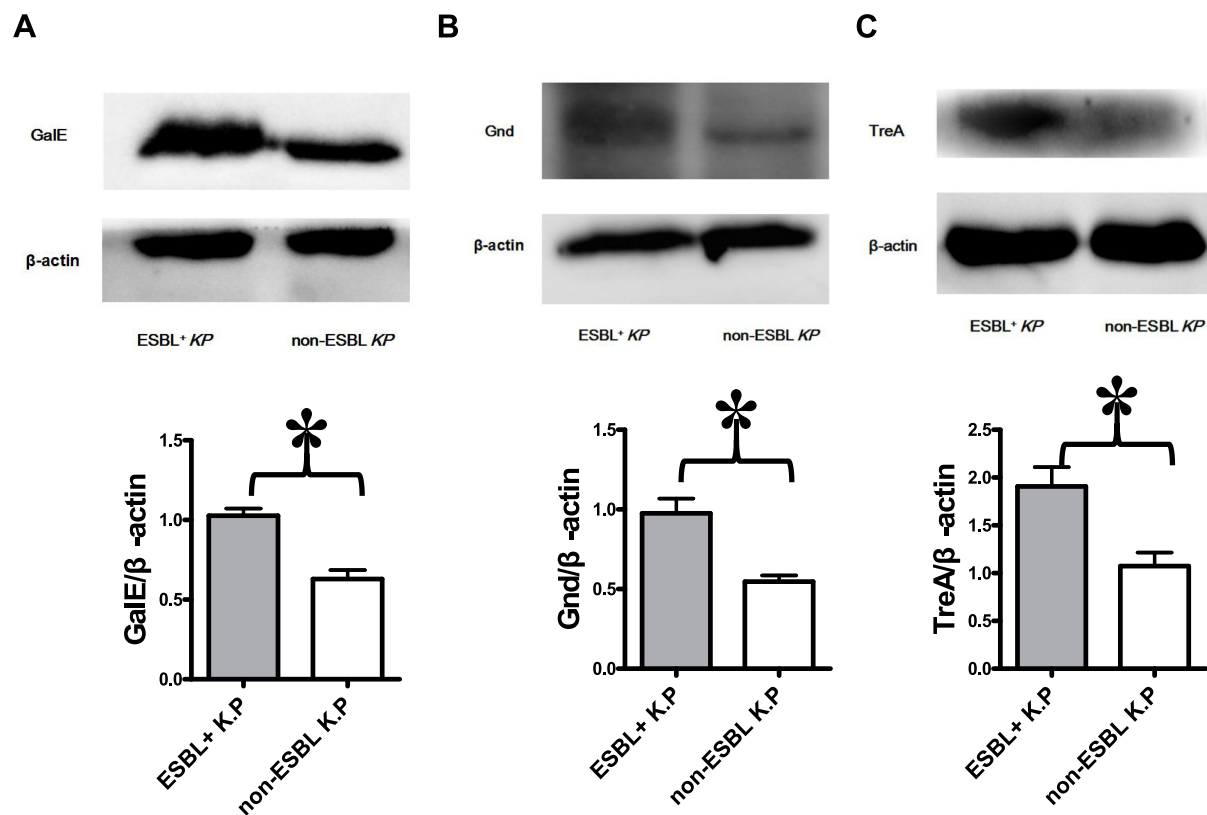
| Accession  | Gene Name   | Coverage    | Peptides | PSMs | Unique Peptides | Abundance B/A | P           |
|------------|-------------|-------------|----------|------|-----------------|---------------|-------------|
| A0A263K3L2 | <i>treA</i> | 63.68330465 | 29       | 142  | 1               | 9.561619718   | 2.07688E-05 |
| A0A193SDM6 | <i>gnd</i>  | 76.28205128 | 35       | 185  | 2               | 6.142857143   | 4.79711E-06 |
| A0A168Y2X7 | <i>sucD</i> | 92.38754325 | 18       | 240  | 1               | 4.268656716   | 6.14267E-06 |
| A0A170CV75 | <i>galE</i> | 76.92307692 | 15       | 64   | 2               | 3.62962963    | 0.000236128 |
| A0A181YC87 | <i>aceE</i> | 75.31003382 | 71       | 517  | 1               | 3.601993865   | 9.49067E-06 |
| A0A0P0YRV3 | <i>rmIC</i> | 45.10869565 | 8        | 27   | 8               | 3.386695906   | 9.08271E-07 |
| A0A024GWD8 | <i>rmID</i> | 52.02702703 | 11       | 18   | 1               | 3.306532663   | 1.72723E-05 |
| A0A1C3SZD7 | <i>rmIA</i> | 44.6366782  | 9        | 16   | 1               | 2.39039548    | 4.56514E-05 |

**Notes:** A=non-ESBL *Klebsiella pneumoniae*; B= ESBL-producing *Klebsiella pneumoniae*.

causing the strong virulence of the ESBL<sup>+</sup> *K. pneumoniae* analyzed here. However, due to the increased antibiotic resistance that it induces, OmpK36 should not be selected as an anti-virulence target for treatment of ESBL<sup>+</sup> *K. pneumoniae* infection.

Our data also reveal a significant increase of galE protein in ESBL<sup>+</sup> *K. pneumoniae* strains. The gene *galE* encodes UDP-glucose 4-epimerase, which epimerizes UDP-galactose and UDP-glucose for galactose metabolism,

as well as LPS and EPS formation, thus influencing both bacterial growth and virulence.<sup>18</sup> When UDP-galactose accumulates, the concentration of UTP or CTP is reduced in cells and the bacterial growth will be cessative.<sup>19</sup> Over-expressed galE has been associated with increased virulence and biofilm formation in numerous bacteria.<sup>20–22</sup> Therefore, disrupting or antagonizing galE may be lethal to ESBL<sup>+</sup> *K. pneumoniae*, highlighting this protein as a promising antimicrobial target.



**Figure 4** Validation of proteomics data by Western blot. The sediments of every 10 isolates of ESBLs<sup>+</sup> *Klebsiella pneumoniae* or non-ESBL *Klebsiella pneumoniae* were mixed as one group of samples. The protein from each group was analyzed for treA, galE and Gnd levels by Western blot. The blots were reprobed with  $\beta$ -actin antibody to confirm equal loading of the samples. Representative Western blot images from three independent experiments for Gale (A), Gnd (B), and TreA (C) levels with  $\beta$ -actin as a loading control are shown. The expression levels of Gale (A), Gnd (B) and TreA (C) are higher in ESBLs<sup>+</sup> *Klebsiella pneumoniae* group. \* $P < 0.05$ .

Capsule, the most thoroughly studied virulence factor, is necessary for *K. pneumoniae* virulence.<sup>23</sup> *Cps* is the conserved gene cluster encoding capsule in *K. pneumoniae* strains, which harbors a number of genes involved in capsule production, including *wza*, *wzb*, *wzc*, *wzi*, *gnd*, *ugd*, *wca*, *cpsB*, *cpsG*, and *galF*.<sup>24–26</sup> In *K. pneumoniae*, *wza*, *wzb*, and *wzc* are involved in *wzy*-dependent K-antigen biosynthesis and export pathway. Our data revealed higher expression of *wza*, *wzb*, *wzc*, *gnd*, and *ugd* in ESBL<sup>+</sup> *K. pneumoniae* strains compared to the non-ESBL strains. The *gnd-ugd* region includes genes involved in the synthesis of GDP-D-mannose (*manB* and *manC*) or dTDP-L-rhamnose (*rmlA*, *rmlB*, *rmlC*, and *rmlD*).<sup>24</sup> Expression of *rmlA*, *rmlC*, and *rmlD* is significantly higher in ESBL<sup>+</sup> *K. pneumoniae* than in non-ESBL strains. Considering that *rml* genes do not exist in human, producing specific dTDP-L-rhamnose biosynthesis inhibitors may directly interfere with ESBL<sup>+</sup> *K. pneumoniae* viability, suggesting the possibility to develop a new class of vaccines targeting nucleotide sugar production against this type of infection. In this study, the expression of *sucD* in ESBL<sup>+</sup> *K. pneumoniae* was also much higher than in non-ESBL strains. *SucD* encodes succinyl-CoA synthase  $\alpha$  subunit. Succinyl-CoA is part of the tricarboxylic acid (TCA) cycle, whose metabolic products also contribute to the synthesis of capsular polysaccharide in *K. pneumoniae*. Capsular polysaccharide production contributes to biofilm formation also in *K. pneumoniae* strains.<sup>27</sup> *SucD* has therefore been identified as an important enzyme in the formation of biofilm in *K. pneumoniae*.<sup>28</sup> Biofilm is a complicated structure comprising bacterial cells embedded in a self-produced EPS matrix,<sup>27</sup> which renders the bacteria less susceptible to antibiotics and enables them to counterattack the host defense. Consequently, inhibiting biofilm formation in drug-resistant *K. pneumoniae* may reduce their resistance to host-mediated defenses<sup>29</sup> and weaken their colonizing ability, thereby increasing their susceptibility to antimicrobial agents.<sup>30</sup>

Nevertheless, there are some limitations of this study. First, the sample size was limited. The number of *K. pneumoniae* strains used is too small to generalize the findings in the study to a large population. Although we have validated some typical differentially expressed proteins between ESBL-producing and non-ESBL-producing *K. pneumoniae* by Western blot analysis, the results still need to be further confirmed in a study with a larger sample size. Second, we have not explored the interaction of these differentially expressed proteins with the immune response in vivo. Further studies focusing on this aspect should be

undertaken, in order to gain new insights to strengthen the treatment of ESBL-producing *K. pneumoniae* infections.

## Conclusion

In conclusion, our proteomic analysis identified at least 9 plausible virulence-associated proteins that represent promising targets for combating ESBL-producing *K. pneumoniae*, particularly *treA*, *galE*, and *gnd*. Moreover, our study also demonstrates that proteomics can offer new avenues for exploring new antimicrobial compounds.

## Data Sharing Statement

The data used and analyzed during the current study are available from the corresponding author on reasonable request.

## Acknowledgments

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## Disclosure

The authors declare that they have no conflicts of interest in this work.

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