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Altered metabolic pathways in classic and hypervirulent *Klebsiella pneumoniae* isolates revealed by proteomics analysis

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Abstract: *Klebsiella pneumoniae* is an opportunistic pathogen that causes a wide range of infections. The emergence and spread of hypervirulent *K. pneumoniae* (hvKp), which appears to be different from the classical *K. pneumoniae* (cKp) in several microbiological aspects, is an urgent global threat. However, the virulence characteristics of hvKp and its differences from cKp are poorly understood. This work aimed to investigate the correlation between the expression characteristics of proteins and hypervirulence, using proteomics. Our results revealed that 185 proteins were upregulated while 266 proteins were downregulated in hvKp isolates when compared with cKp isolates. The differentially expressed proteins were functionally categorized according to the Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. These proteins were predominantly metabolism associated, which indicates that changes in the metabolic pathways in hvKp isolates might in part contribute to hypervirulence.

Keywords: *Klebsiella pneumoniae*; virulence; proteomics; metabolic pathway

INTRODUCTION

Klebsiella pneumoniae is a nonmotile, encapsulated, Gram-negative bacillus prevalent in the environment that also colonizes human mucosal surfaces [1]. It is an opportunistic pathogen that causes a wide range of infections, especially in diabetics and alcoholics. Typically, classical *K. pneumoniae* (cKp) strains cause respiratory and urinary tract infections, bacteremia and meningitis [2]. The emergence and spread of hypervirulent *K. pneumoniae (*hvKp*)* are serious problems [3]. A well-known virulent pathogen, hvKp contains several virulence genes located on the chromosome and plasmids that confer its hypervirulent phenotype [4]. The hvKp strain appears to be different from the cKp in a number of microbiological aspects. Several classical traits have been associated with hvKp as compared to cKp, such as a hypermucoviscous phenotype, a positive “string” test, the predominance of K1 and K2 capsule types, and multiple virulence genes [5,6]. In addition, unlike infections of cKp, hvKp can cause highly invasive infections such as liver abscesses in both immunocompromised and healthy individuals [7]. However, the virulence and pathogenic characteristics of hvKp and its differences from cKp are less well understood.

Comparative proteomic analysis has been extensively used to identify presumed bacterial virulence factors [8], revealing differences in protein abundance between high and low virulence isolates. These results could help to elucidate the virulence factors in bacteria [9]. Proteomic studies have been performed in *K. pneumoniae* in response to environmental factors like metal and antibiotic stress [10-12]. In the present work, the clinical isolates of cKp and hvKp were collected from patients in Baotou, China. Comparative proteomics was applied to explore the correlation between the expression characteristics of proteins and hypervirulence. A total of 451 differentially expressed

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proteins were identified, and further bioinformatic analysis showed that these proteins were predominantly metabolism associated. The obtained results will help create a novel strategy for developing antibacterial drugs against hvKp.

MATERIALS AND METHODS

Ethics statement

Ethical approval was obtained from Baotou Medical College Research and Ethics Review Committee. Informed written permission was obtained from participants. Any data generated from the specimens protected patient privacy, confidentiality and anonymity.

Bacterial strain collection and drug susceptibility testing

The cKp and hvKp isolates were collected from the microbiology laboratory of the First Affiliated Hospital of Baotou Medical College in Baotou, China. The criterion for inclusion of hvKp isolates (n=6) was whether they were isolated from patients with a clinical syndrome of hepatic-invasive infection. Since most *K. pneumoniae* infections in the local area are presumably due to cKp strains, the cKp isolates (n=6) were generated from randomly chosen deidentified blood isolates. The isolates of *K. pneumoniae* (cKp and hvKp) were confirmed through standard microbiological and biochemical characterization methods [13]. Antimicrobial susceptibility testing was performed using the disk diffusion method according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute (CLSI) [14].

Sample preparation

Bacterial cells were collected, washed in ice-cold phosphate-buffered saline (PBS) and subjected to protein extraction using lysis buffer (8 M urea, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail, followed by 3 min of sonication on ice using a high-intensity ultrasonic processor (Scientz, China). After quantifying the protein concentration by the Bradford method [15], the extracted protein was digested with trypsin digestion buffer (PH 8.0) at 37°C for 2 h at 300 ×g with shaking. The samples were then extracted with 50% acetonitrile and 0.1% formic acid following desalination as described previously [15]. Peptides were lyophilized in a vacuum concentrator (Thermo Fisher Scientific, MA, USA).

Liquid chromatography coupled with tandem mass spectrometry analysis

The Evosep One liquid chromatography system (Evosep, Odense, Denmark) was connected to quadrupole time-of-flight mass spectrometry (Bruker Daltonics, Billerica, MA, USA). Peptides were reconstituted in 0.1% formic acid and separated on a 15-cm analytical column (150-µm inner diameter) with a 60-min gradient. The column temperature was maintained at 50°C. During parallel accumulation-serial fragmentation (PASEF) tandem mass spectrometry (MSMS) scanning, the collision energy was ramped linearly as a function of the mobility from 59 eV at 1/K0=1.6 Vs/cm^2 to 20 eV at 1/K0=0.6 Vs/cm^2.

Data analysis

Raw files were processed and analyzed by Spectronaut version 15.0 (Biognosys AG, Switzerland) at default settings. Spectronaut was set up to search the database of Uniprot-*Klebsiella_pneumoniae_*subsp. *pneumoniae* (version 202109, 5126 entries). Carbamidomethyl (C) was specified as the fixed modification, and oxidation (M) was specified as the variable modification. The significantly differentially expressed proteins (DEPs) were selected if their absolute fold change was >1.5 and P value <0.05 (bilateral Student's t-test).

Bioinformatic analysis

Principal component analysis was carried out separately on each data set using the R package "gmodels" (version 2.18.1) with default parameters. A volcano plot was carried out using the ggplot2 package (http://ggplot2.org). Blast2GO version 5 was used for functional annotation and GOATOOLS was used to perform Gene Ontology (GO) enrichment analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was processed by KOBAS (http://kobas.cbi.pku.edu.cn/). Enrichment significance was determined using Fisher's exact test.

RESULTS

Antimicrobial susceptibility

Of the 9 antibacterial agents tested, the frequencies of resistance of cKp isolates to tetracycline, chloramphenicol, cotrimoxazole, piperacillin, cefazolin, cefotaxime, ciprofloxacin, gentamicin and ceftazidime were 42.2%, 42.2%, 41.3%, 41.3%, 37.0%, 35.7%, 27.0%, 24.0% and 21.3%, respectively. In contrast, the frequencies of resistance of cKp isolates to tetracycline, chloramphenicol, cotrimoxazole, piperacillin, cefazolin, cefotaxime, ciprofloxacin, gentamicin and ceftazidime were 14.6%, 16.6%, 18.7%, 18.7%, 18.7%, 35.7%, 18.7%, 10.4% and 10.4%, respectively. There was a significant difference in the resistance to these 9 antibacterial agents tested between cKp and hvKp isolates ($P<0.05$). All strains were sensitive to imipenem and meropenem (Supplementary material Table S1).

Pattern characteristics of cKp and hvKp strains

To search the protein patterns of different *K. pneumoniae* clinical isolates, a total of 12 *K. pneumoniae* strains, including six cKp and six hvKp isolates obtained from 12 patients were subjected to LC-MS/MS. As a result, 33,954.2 peptides (30,056-35,444, Fig. 1A) and 3,191.9 protein groups (3,089-3,256, Fig. 1B) per sample were detected and quantified on average. Ranked proteins in cKp and hvKp groups indicated similar distributions (Fig. 1C, D). Score plots of PCA and OPLS-DA showed that hvKp isolates could be separated from cKp isolates (Fig. 2A, B).

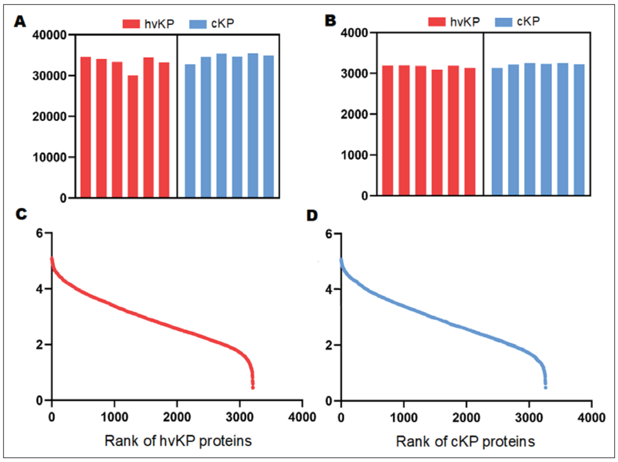

Fig. 1. Identified and quantified peptides and proteins. **A** – Identified and quantified peptides. **B** – identified and quantified proteins. **C** – The rank of quantified proteins in the hvKp group. **D** – The rank of quantified proteins in the cKp group.

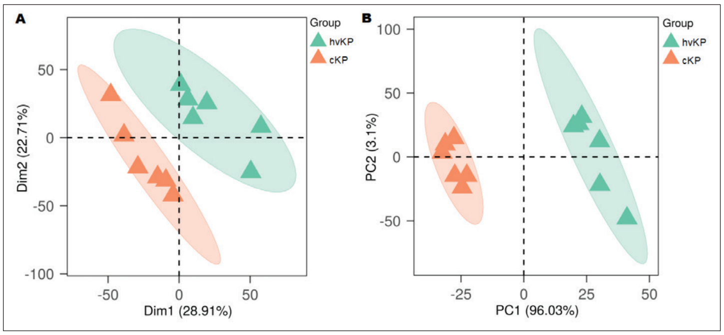

Fig. 2. Score plots of PCA and OPLS-DA. **A** – PCA; **B** – OPLS-DA.

Proteomics-based analysis revealed differently expressed proteins in hvKp and cKp isolates

The expression values of proteins in hvKp isolates and cKp isolates were compared. We observed that 451 known proteins were significantly differentially expressed ($P<0.05$), of which 185 were significantly upregulated and 266 were significantly downregulated. Fig. 3 shows the volcano plot of differentially expressed proteins. The top 20 upregulated proteins included vitamin B12-transporter protein precursor (BtuF), putative enzyme, thiamine biosynthesis protein (ThiF), peptidoglycan lytic exotransglycosylase and peptidyl-prolyl *cis-trans* isomerase C (Table 1).

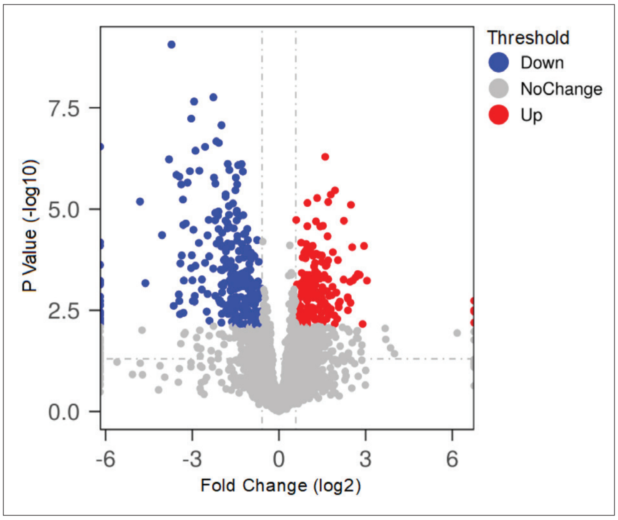

Fig. 3. Volcano plot of differentially expressed proteins between the cKp group and the hvKp group.

Table 1. Top 20 proteins with increased expression in the hvKp group as compared to the cKp group.

Description	Gene name	Fold change	P-Value
Vitamin B12-transporter protein BtuF	KPN_03089	8.258791676	0.000584613
Uncharacterized protein	KPN_02025	7.702315513	8.13778E-05
Putative enzyme	ycfS	7.440467124	0.006890519
Thiamine biosynthesis protein ThiF	thiF	6.940456419	0.000422905
Peptidoglycan lytic exotransglycosylase	mltD	6.625998167	0.000403675
Peptidyl-prolyl cis-trans isomerase C (Rotamase C]	ppiC	6.311429008	0.000483244
D-tagatose-1,6-bisphosphate aldolase subunit GatZ	gatZ	5.886356336	0.000575328
S-adenosylmethionine-dependent methyltransferase	KPN_03088	5.797794071	8.71903E-05
Putative methionine synthase	KPN_01615	5.622465121	7.87421E-06
Putative transport protein, PTS system	KPN_00551	5.56693659	0.002062187
Sulfur carrier protein ThiS	thiS	5.516577981	0.00062479
Outer membrane porin protein, putative ferrisiderophore receptor	ybiL	5.2322538	0.00317074
Uncharacterized protein	KPN_01676	5.210853923	0.001613906
Putative enzyme with dioxygenase domain	ygiD	5.207938137	0.001505023
Putative glyoxalase/bleomycin resistance protein/dioxygenase	KPN_01697	4.750027283	1.9431E-05
Hypothetical isochorismatase family protein	KPN_00318	4.71359697	0.00055145
Thiamine-phosphate synthase	thiE	4.309149356	0.001923339
Isochorismatase	entB	4.204610755	0.002157751
Isochorismate synthase	entC	4.200060722	0.00265437
Putative PTS family enzyme IIA component	KPN_04197	4.174508028	0.002208081

Table 2. Top 20 proteins with decreased expression in the hvKP group as compared to the cKP group.

Description	Gene name	Fold change	P-Value
Iron transporter: fur regulated	sitA	-27.99966722	6.52378E-06
ATPase with chaperone activity, ATP-binding subunit	KPN_pKPN3p05899	-24.63794303	0.000675778
3-oxoadipyl-CoA thiolase	paaJ	-16.51446317	4.3999E-05
ABM domain-containing protein	KPN_01147	-13.96127196	5.92706E-07
Putative oxidoreductase	KPN_01842	-13.15719495	8.65663E-10
Alpha-galactosidase	melA	-12.48706551	0.002458788
Probable enoyl-CoA hydratase	paaF	-11.67586166	1.42422E-06
Putative homeobox protein	ybgS	-11.04765572	0.001286294
4-hydroxyphenylacetate catabolism	hpaB	-10.91590965	1.58401E-06
Glutarate 2-hydroxylase	csiD	-10.90897144	0.001871909
Dihydropteroate synthase	sul	-10.7371665	0.003944661
Cation/acetate symporter ActP	actP	-10.63347992	0.000220023
Enoyl-CoA hydratase	paaG	-10.41586568	2.46015E-06
Glutamate/aspartate transport protein (ABC superfamily, membrane]	gltK	-10.29265225	0.000139838
High-affinity branched-chain amino acid transport protein (ABC superfamily, peri_bind]	livJ	-9.999641038	5.8195E-06
3-hydroxybutyryl-CoA dehydrogenase	paaH	-9.923217421	2.47176E-05
Methylated-DNA--[protein]-cysteine S-methyltransferase	ada	-9.873036932	0.003651078
sn-glycerol 3-phosphate transport protein (ABC superfamily, peri_bind]	ugpB	-9.79262294	0.000581682
N-succinylglutamate 5-semialdehyde dehydrogenase	astD	-9.345822424	2.25888E-05
Succinylglutamate desuccinylase	astE	-8.95598986	2.22587E-06

The top 20 downregulated proteins are listed in Table 2, including ferric uptake regulator (Fur) protein, ATPase with chaperone activity, 3-oxoadipyl-CoA thiolase, ABM domain-containing protein and putative oxidoreductase.

Functional categorization of differentially expressed proteins

Next, we classified the upregulated and downregulated proteins based on the relative categories annotated by the GO method. Among the differentially expressed proteins classified as being involved in biological processes, 198, 129 and 152 proteins were classified into the categories of organic substance metabolic process (GO:0071704; P-value=4.93E-06), small molecule metabolic process (GO:0044281; P-value=2.85E-06) and primary metabolic process (GO:0044238; P-value=1.91E-03), respectively (Fig. 4A). For molecular function, 135, 109 and 94 proteins were classified into the categories of ion binding (GO: 0043167; P-value=1.38E-03), transferase activity (GO: 0016740; P-value=1.88E-03) and hydrolase activity (GO: 0016787; P-value=4.30E-02), respectively, which were catalytic activity and binding related (Fig. 4B). Moreover, for cellular components, the majority were intracellular-related.

KEGG pathway analysis of differentially expressed proteins

The functions of the differentially expressed proteins were further analyzed by the KEGG pathway annotation method and 88 pathways were obtained. Only 24 enriched pathways had a P-value of <0.05. The top 20 downregulated proteins are listed in Fig. 5, including metabolic pathways (P-value=3.34E−09), microbial metabolism in diverse environments (P-value=2.06E−07) and biosynthesis of secondary metabolites (P-value=1.97e−03).

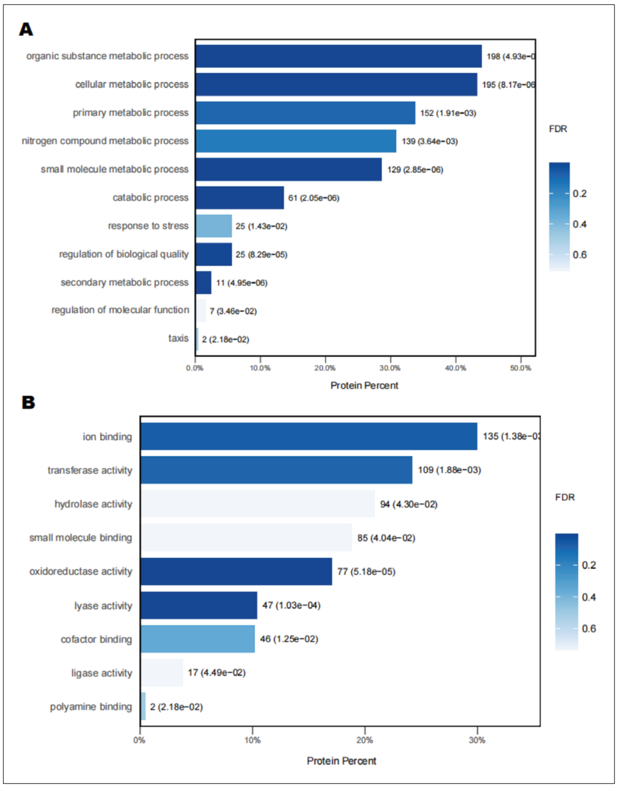

Fig. 4. GO enrichment analysis of differentially expressed proteins between the cKp and hvKp groups. The x-axis represents the GO terms of biological processes (**A**) and molecular functions (**B**). The y-axis represents the number and percentage of proteins.

DISCUSSION

Current therapeutic options for *K. pneumoniae* infection have become limited due to its ability to acquire additional antibiotic resistance genes or virulence genes [16]. The hvKp, which is more virulent than cKp, has emerged as a pathogen of global concern [17]. To date, however, relatively little is known about the molecular pathogenesis of hvKp. A previous study applied comparative proteomics to explore the correlation between pattern characteristics and drug resistance. The results showed that metabolic pathway alteration was directly correlated with the antibiotic resistance of *K. pneumoniae* [15]. In this study, we used quantitative proteomic analysis to investigate the correlation between the proteomic pattern and hypervirulence of hvKp.

Our results showed that several proteins that were upregulated might play a role in the hypervirulence. These proteins include vitamin B12-transporter protein BtuF, uncharacterized protein KPN_02025, putative enzyme ycfS, thiamine biosynthesis protein ThiF and peptidoglycan lytic exotransglycosylase MltD. BtuF is responsible for the uptake of vitamin B12 across the bacterial inner membrane [18]. It was shown that B12 biosynthesis is an important step in the metabolism of bacterial species during host

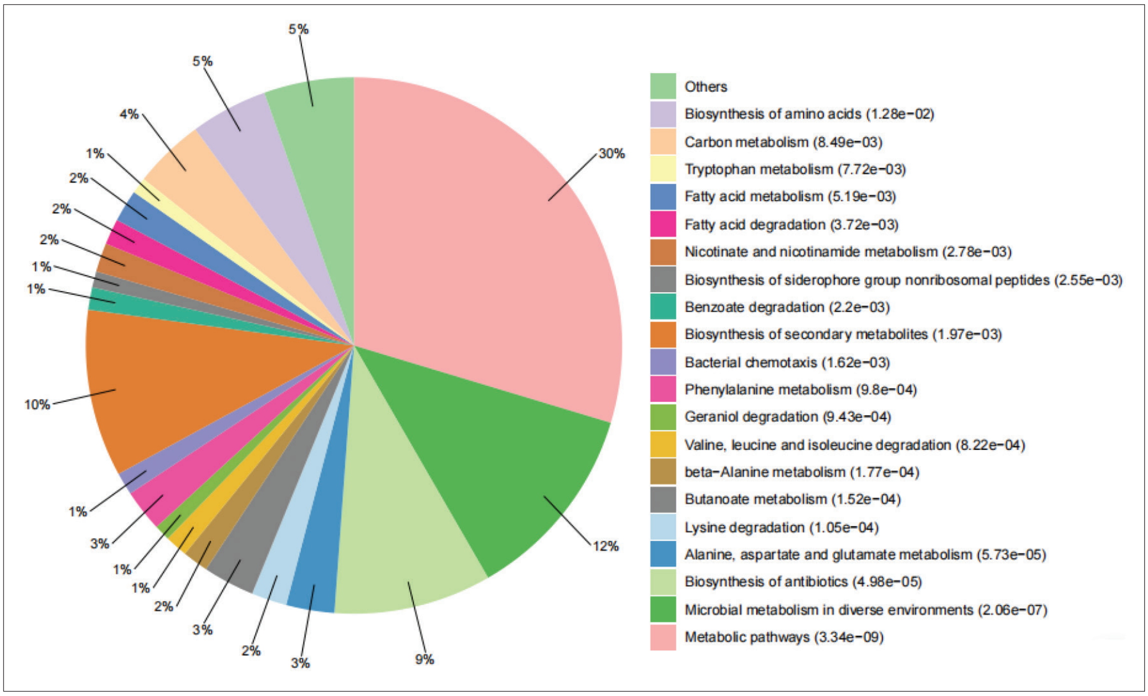

Fig. 5. KEGG enrichment analysis of differentially expressed proteins between the cKp and hvKp groups.

infection [19,20]. Thus, upregulated expression of BtuF might be involved in the virulence of hvKp. The putative enzyme ycfS is involved in the attachment of the Braun lipoprotein to peptidoglycan, which has important roles in cell division, cell daughter separation and maintenance of bacterial morphology [21]. Similarly, MltD plays a key role in bacterial morphology, growth and peptidoglycan metabolism. A previous study also reported that MltD possibly has a virulence-related function [22]. ThiF is an enzyme involved in the biosynthetic cascade for generating the essential cofactor thiamin pyrophosphate, which is involved in the metabolism of carbohydrates and the biosynthesis of branched-chain amino acids [23]. These proteins play key roles in modulating bacterial metabolic state, which is intimately connected to its pathogenesis [24].

Several proteins were downregulated, including ferric uptake regulator (Fur) protein sitA, ATPase with chaperone activity KPN_pKPN3p05899, 3-oxoadipyl-CoA thiolase paaJ, ABM domain-containing protein KPN_01147 and putative oxidoreductase KPN_01842. A recent report showed that the acquisition of ferrous iron [Fe^{2+}] from the environment is an important virulence factor utilized by *K. pneumoniae* to establish infection in human hosts [25]. A relatively high concentration of Fe^{2+} of hvKp could repress sitA, and this regulation was Fur-dependent [26]. However, a previous study also found that sitA contributes to the virulence of *K. pneumoniae* in a mouse infection model [27]. Its effect on bacterial virulence needs further study. Similarly, Fur senses iron sufficiency and represses genes that enable iron uptake, such as haem-degrading monooxygenases that contain the typical ABM domain [28]. Therefore, we assumed that the ABM domain-containing protein KPN_01147 was repressed by Fur in hvKp isolates. ATPase with chaperone activity has a critical role in mediating bacterial drug resistance [29]. We found that cKp isolates have a higher drug resistance rate than hvKp. This might be the reason why the expression of ATPase with chaperone activity is downregulated in hvKp. The paaJ-regulated fatty acid β oxidation is the major pathway for the degradation of fatty acids and is essential for maintaining energy homeostasis in bacteria [30]. It is well known that host-derived fatty acids are used as an important carbon source. However, the overactive catabolism of fatty acids is detrimental to mycobacterial cell growth and pathogenicity [31]. Thus, we supposed that fatty acid catabolism of hvKp is repressed by paaJ to sustain virulence; it has been reported that this type of metabolism likely affects bacterial virulence [32]. We suspect that putative oxidoreductase KPN_01842 might enhance hvKp virulence via alteration of fermentative metabolism.

To understand the gene functions associated with the differentially expressed proteins, GO enrichment analysis and KEGG pathway analysis were performed. The GO enrichment analysis indicated that the differentially expressed proteins were predominantly metabolism associated. KEGG pathway analysis predicted that the differentially expressed proteins are associated with metabolic pathways, microbial metabolism in diverse environments and biosynthesis of secondary metabolites.

CONCLUSIONS

Proteomics was performed on cKp and hvKp clinical isolates. A total of 451 proteins were identified. Further bioinformatic analysis revealed that the differentially expressed proteins lead to downstream consequences resulting in alteration of metabolic pathways, microbial metabolism in diverse environments and biosynthesis of secondary metabolites, which may contribute to sustaining virulence and cell survival in hvKp isolates.

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Author contributions: HY conducted the study, collected the data, performed the analysis of data and prepared the manuscript. LZ and RS performed the experiments. HH and ZW designed the study and edited the manuscript. All authors approved the final version of the manuscript.

Conflict of interest disclosure: The authors declare that there is no conflict of interest.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Yu%20et%20al_%207846_Data%20Report.pdf

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Supplementary Data

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Yu%20et%20al_7846_Supplementary%20Material.pdf