Proteomic profiling of salmonella under berberine stress

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Abstract. Drug resistance of pathogen raises severe problems these decades since the antibiotic abuse. The need to explore the adaption mechanism of pathogens to antibiotic and develop new drugs is urgent. In this experiment, we explore the proteome of Salmonella enterica serovar Typhimurium under stress of berberine to study the adaption mechanism of S. Typhimurium to berberine. After proteomic analysis, we found that energy metabolism-related proteins and flagellar proteins were up-regulated and ribosome proteins were down-regulated under berberine pressure, revealing the potential mechanisms of growth inhibition of berberine to Salmonella. The possible inhibiting mechanism include three parts: 1. The metabolism of S. Typhimurium was disturbed by Berberine; 2. Berberine has a negative effect on ribosomes of S. Typhimurium; 3. Berberine destroys the flagella of S. Typhimurium.

1. Introduction

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a pathogenic bacterium of enterobacteriaceae, which lives a parasitic life of facultative anaerobic. This Gram-negative bacterial pathogen can infect both human and other animals and cause acute digestive tract disease. After oral ingestion through contaminated food, S. Typhimurium can cause infection. S. Typhimurium is able to survive in gastric acidity through its enzymes and cross intestinal epithelium. Food poisoning can occur when food contaminated by people infected with salmonella or the faeces of carriers. According to statistics, among all kinds of bacterial food poisoning in the world, food poisoning caused by salmonella always ranks the top.

Natural products is always a treasure trove for antibiotic. Many natural products from Chinese herbal medicine show good antibacterial activity. As a alkaloid extracted from plants such as Coptis chinensis and Phellodendron amurense Rupr, berberine has a significant antibacterial effect, which has been used to treat digestive diseases such as bacterial gastroenteritis and dysentery for a long time. Recently, some works shows that berberine has a good clinical effect on illnesses caused by Salmonella. However, the effective dose of berberine is obviously higher than other traditional antibiotic such as streptomycin and the molecular mechanism of its antibacterial activity remains to elicited.

Proteins are the executors of life activities. Not only the activity but also their abundance influence the living state of an organism. The development of mass spectrometry allows us to profile the abundance of proteins in high throughput. Since human genome project, proteomics has many

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applications in current scientific research. We designed the experiment with the help of the theory of
proteomics.[1]

We set the protein as the main target which is the actor of physical activity and use the technique of
proteomics to analyze the changes of protein group data between S. Typhimurium processed by
berberine and S. Typhimurium without berberine to explore the effects of the berberine on
salmonella. We found that berberine inhibits S. Typhimurium by disrupting energy metabolism of S.Ty
phimurium, inhibiting flagellum synthesis, and destroying ribosomes.

2. Materials & Methods

2.1. Bacteria cultivation and molecular cloning

Salmonella enterica serovar Typhimurium strains (SL1344) were stored for long period under
–80°C condition in 40% glycerol. When used, the frozen bacteria were cultivated first on agar plates at
37°C. After about 12 hours, the single colony was appeared and was picked for further growth in 3 mL
LB medium. Another 12 hours later, the culture was diluted in 1:20 ratio into new LB medium and
grown to reach mid-exponential phase (OD600 = 0.9).

2.2. Treatment of berberine and sample preparation

Salmonella was raised in LB medium overnight before berberine (Sigma) treatment. After
overnight culture, the culture was diluted with ratio 1:20 into new LB medium which contains
corresponding dose of berberine. At indicated time points, the viable bacteria in the medium were
numerated by colony-forming unit (CFU) assays. After 3 hour culture, the medium were spun at 4000
× g for 20 min to pellet intracellular bacteria. Bacterial pellets were washed with wash buffer (25 mM
Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) to
remove residual contaminants. The bacterial samples in pellet were resuspended and denatured in 1 ×
SDS-PAGE loading buffer at 95°C for 15 min during which the bacteria was lysed. The SDS-PAGE
samples were separated using 8% SDS-PAGE. The resulting gel was stained with coomassie blue and
cut into 8 gel bands for further in-gel digestion. The resulting peptides in gels were extracted with
0.1% FA. The extracted solution and the trypsin solution were collected, vacuum dried and
resuspended in 3% acetonitrile for proteomic analysis.

2.3. LC-MS/MS proteome analysis

LC-MS/MS experiments were conducted on nano-flow reversed-phase liquid chromatography
(EASY-nLC 1000, Thermo Scientific) coupled tandem ion trap Orbitrap mass spectrometer (LTQ-
Orbitrap Velos, Thermo Scientific). Home-made column (75 μm × 150 mm) was filled with C-18
modified silica-based particles (Michrom BioResources Inc., Auburn). BSA was used as quality
control sample. The gradient was about 4 min with solvent B (95% ACN and 0.1% FA) changing from
5% to 45%. The electrosprayed peptides was detected in MS and MS/MS analyzes with data-
dependent acquisition mode. Three biological replicates were conducted.

2.4. Proteomic data analysis and label-free quantification

MaxQuant (Version 1.5.3.30) was used to analyze the raw MS files. Andromeda was used for
database matching with following parameters: precursor mass tolerance was 20 ppm, MS/MS
fragment mass tolerance was 0.8 Da and two maximum missed cleavages was allowed. Carbamidomethyl on Cys modification was set as a fixed modification and oxidation of Met was set as a
variable modification. Acquired MS/MS spectra were searched against an S. enterica serovar
Typhimurium SL1344 protein database (downloaded from UniProt) augmented with the human
protein database. The false discovery rate (FDR) of peptides and proteins was controlled at < 1%. The
MaxQuant software was used to calculate the label-free quantitation (LFQ) intensity for each protein,
which was further processed using the Perseus software (version 1.5.4.1). Logarithmic values (Log2)
of the LFQ intensity were used and the missing values were replaced with random numbers from a
normal distribution (width = 0.3, shift = 1.8).
3. Results & discussions

3.1. Dose dependent growth inhibition of berberine to Salmonella

First, we investigated the dose effect on the growth inhibition activity of berberine to S. Typhimurium. As figure 1 showed, the growth inhibition of berberine is obviously dose-dependent as S. Typhimurium growth rate in LB medium containing 10 mg/mL berberine did not show significant different to that in LB medium. However, as the concentration of berberine rose up to 50 mg/mL, the growth rate of S. Typhimurium is significant suppressed. Thus, we chose the S. Typhimurium grew under 50 mg/mL berberine as the sample for proteomic study for the fact that the berberine start to influence the growth of S. Typhimurium but did not totally disrupt the growth of S. Typhimurium.

![Figure 1](image)

**Figure 1.** The effect of berberine dose on S. Typhimurium growth in LB medium.

3.2. Proteomic overview of Salmonella under berberine stress

In the experiment, we detected a total of 1,007 proteins. Among them, there were 413 proteins whose expression levels were up-regulated (more than 2 fold) compared with the control groups and 369 proteins that were down-regulated by more than 0.5 fold. With the help of KEEG, UNPROT, ECOCYC database, we explore the relationship between these regulated proteins whose expression were up-regulated or down-regulated and the adaption mechanism of S. Typhimurium under berberine stress. After deep sorting, we find out that the berberine influent S. Typhimurium growth through several mechanism related to different pathway including metabolism, peptide synthesis and recycle.

3.3. The metabolism of S. Typhimurium was disturbed by Berberine

Compared to the control group, the expression level of the protein related to energy supply increased in the experimental group. The pathways involved in these proteins include anaerobic respiratory pathway, galactose metabolism, glycolysis pathway, glucosamine metabolism, pyruvate metabolism, nicotinamide metabolism, amino acids (including glycine, serine, urehan, arginine, proline) metabolism, purine metabolism and pyrimidine metabolism. The abundance of proteins associated with anaerobic respiration, such as TorA, TorD, TorC, SerS, SerA and SerC increased significantly by 290 fold, 14 fold, 110 fold, 48 fold, 26 fold, 2.125 fold. As torA encodes an inducible
trimethylamine N-oxide reductase, TorA receives electrons from the membrane bound cytochrome c menaquinol dehydrogenase TorC. TorC is a pentaheme c-type cytochrome that is anchored to the inner membrane. The ability of TorC to transfer electrons from the membrane's quinoline to TorA in the cytoplasm provides the basis for anaerobic bacterial growth. TorD is the dedicated chaperone of TorA. TorD is involved in the protection of TorA. torC, torA and torD form an operon, which is regulated by the TorTSR two component system. Expression is induced by trimethylamine N-oxide (TMAO) and repressed by oxygen. TorCAD is expressed during exponential growth with TMAO under both anaerobic and aerobic conditions and TMAO can be reduced during aerobiosis. The TorC apolipoprotein is involved in the automatic regulation of the tor operon. Thus, it's obvious that the berberine influence the anaerobic pathway of S. Typhimurium.

The enzyme is regulated by allosteric end-product inhibition that shows cooperativity. Inhibition by serine acts primarily through reduction of catalytic velocity and has only a small effect on the Kms of the substrates. SerA is thus classified as a type V allosteric enzyme. The serC-encoded enzyme, phosphoserine/phosphohydroxythreonine aminotransferase, functions in the biosynthesis of both serine and pyridoxine, by using different substrates. In addition, some proteins associated with galactose metabolism like GalM, GalK, MelA are up-regulated by 18 fold, 15 fold, 19 fold. GalM is involved in the subpathway that converts alpha-aldose to the beta-anomer. Galactokinase (GalK) catalyzes the first step in the Leloir pathway of galactose metabolism. α-galactosidase (MelA) is a dimer that required for utilization of α-galactosides as nutrients. MelA activity can be induced by three α-D-galactosides: melibiase, melibitol and galactinol. The enzymatic activity is very unstable upon purification, but can be stabilized by the addition of NAD. PMM/PGM plays an important role in the conversion of 6-phosphate sugar. There are a lot of upregulated proteins like LpdA, PykA, GpmI, STM1627, AldB, Pgk, RreA that are 3.4 fold, 8 fold, 12 fold, 22 fold, 40 fold, 2.9 fold and 29 fold involved in glycolysis. All of these proteins play an important role in the glycolytic pathway. Therefore, the berberine also influence the glycolytic pathway, which may be the reason for the growth inhibition.

Lipid amide dehydrogenase (LpdA) is involved in the formation of glycine cleavage system as well as of the alpha-ketoacid dehydrogenase complexes. Pyruvate kinase II (pykA) is involved in step 5 of the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate. 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (GpmI) is involved in the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate.

S-(hydroxymethyl)glutathione dehydrogenase having an environment where high formaldehyde dehydrogenase activity requires the presence of glutathione, and catalyzing the oxidation of normal alcohols does not depend on glutathione. Aldehyde dehydrogenase B (AldB) catalyzes the NADP-dependent oxidation of diverse aldehydes. Its preferred substrates are acetaldehyde and chloroacetaldehyde. Phosphoglycerate kinase (Pgk) is involved in the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate.

Periplasmic trehalase (TreA) breaks down trehalose into glucose, allowing cells to use trehalose at high osmotic pressures.

GlmM, GlmU, WecB, MurA, YbhC that involved in glucosamine metabolism increased 19 fold, 4.7 fold, 11 fold, 10 fold, 68 fold respectively.

Phosphoglucomutase (GlmM) is involved in the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate. Bifunctional protein (GlmU) is involved in the synthesis of UDP-N-acetyl-alpha-D-glucosamine e and N-acetyl-alpha-D-glucosamine 1-phosphat.

UDP-N-acetylgalactosamine 2-epimerase (WecB) play an important role in catalyzing the reversible epimerization at C-2 of UDP-N-acetylgalactosamine (UDP-GlcNAc) and thereby provides bacteria with UDP-N-acetylmannosamine (UDP-ManNAc), the activated donor of ManNAc residues. Also involved in bacteriophage N4 adsorption. UDP-N-acetylgalactosamine 1-carboxyvinyltransferase (MurA) is involved in the pathway peptidoglycan biosynthesis, which is part of Cell wall biogenesis.

FumA, FumC, AccC, AckA that involved in pyruvate metabolism increased 2.9 fold, 100 fold, 8.3 fold, 6.3 fold. Fumarate hydratase class I, aerobic (FumA) catalyzes the reversible hydration of fumarate to (S)-malate. In the citric acid cycle, it functions as an aerobic enzyme in the direction of
malate formation \cite{14}. Under conditions of iron limitation and oxidative stress, fumarate hydratase class II (FumC) can be a backup enzyme for FumA. It is related to the stereospecific interconversion of fumarate to L-malate\cite{15}. AccC is a component of the acetyl coenzyme A carboxylase complex \cite{16}. Acetate kinase (AckA) catalyzes the formation of acetate phosphate from acetate and ATP, and the reverse reaction can also be catalyzed. This enzyme is also involved in the synthesis of most of the ATP that formed catabolically during anaerobic growth of the organism\cite{17}.

SthA, STM4519, PntA, AtpA and AtpD that involved in nicotinamide metabolism increased 52 fold, 13 fold, 4 fold, 4.6 fold, 3 fold. Soluble pyridine nucleotide transhydrogenase (SthA) is linked with NADPH. While NAD(P) transhydrogenase subunit alpha (PntA) play a role in the transhydrogenation from NADH to NADP, which coupled to ATP hydrolysis and energy production. It is a proton pump across the membrane\cite{18}.

GlyA, SdaB, SdaA, DegQ, DegP, TdcG, TdcC, ThrC, IlvC that involved in amino acids metabolism increased 6.9 fold, 34 fold, 7.5 fold, 50 fold, 14.7 fold, 54 fold, 22 fold, 22 fold and 26 fold. The primary source of the single-carbon group required for biosynthesis of purines, thymine, methionine, and other important biomolecular molecules depends on the catalytic conversion of serine and glycine to each other using tetrahydrofolate (THF) as a single-carbon carrier\cite{19}.

As possible stress response of \textit{S. Typhimurium}, many pathways which could produce energy for \textit{S. Typhimurium} is severely up-regulated. However, there are some unexplained aspects, such as the increase in expression of proteins associated with methane metabolism and galactose metabolism. Galactose metabolism and methane metabolism are the natural metabolic systems of \textit{S. Typhimurium}, which is a symbol of facultative anaerobic bacteria. However, in the nutrient environment we provide, \textit{S. Typhimurium} seems have no need to activate these two metabolic pathways to get energy because we don't supply \textit{S. Typhimurium} with any nutrient which is rich in or can be broken down into galactose and we supply adequate oxygen to \textit{S. Typhimurium}. Thus \textit{S. Typhimurium} is not able to get energy through galactose metabolism and methane metabolism. The energy used to express the proteins involved in methane and galactose metabolism has become a pointless waste. To sum up, we believe that berberine can disrupt the metabolism of \textit{S. Typhimurium}, which is one of the important causes of \textit{S. Typhimurium} death.

### 3.4. Berberine has a negative effect on the ribosomes of \textit{S. Typhimurium}

Most of the 50S ribosomal protein RplR, RplS, RplW, RpmA, RpmB, RpmC and 30S ribosomal protein RpsH, RpsO, RpsQ were down-regulated. These proteins significantly related to the most important biological process: rRNA binding and structural constituent of ribosome\cite{20}. Ribosomes are very important organelles in the translation process of all cell organisms whose number is relatively abundant and constant under normal circumstances. When there are many translation tasks, the number of ribosomes will increase. In this experiment, however, ribosome numbers dropped significantly. Excluding the fact that \textit{S. Typhimurium}’s metabolic system is disrupted by berberine, the number of ribosomes in the experimental group should be the same as that in the control group. Therefore, it is reasonable to assume that berberine has a negative effect which may is destroying ribosome structure and promoting the digestion of proteases and nucleases on the ribosome system of \textit{S. Typhimurium}. Meanwhile, after addition of berberine to the experimental group, the expression of various amino acids-tRNA transferase increased, which may be a sign that the \textit{S. Typhimurium} remedy the ribosome system’s breakdown. The breakdown of ribosome system leads to \textit{S. Typhimurium}’s death.

### 3.5. Berberine destroys the flagella of \textit{S. Typhimurium}

Flagella is very important to the lives of \textit{S. Typhimurium}, allow the \textit{S. Typhimurium} to move and, more importantly, attach itself to the surface of the host cell. Flagellin (FlIC) and Flagellar hook-associated protein 3 (FlgL) have structural molecule activity\cite{21}. Flagellar motor switch protein FlIM has motor activity. FlIG has identical protein binding and motor activity. Regulator of sigma S factor FlIZ has core promoter binding and sigma factor antagonist activity\cite{22}. Flagellar hook protein FlgE has bacterial-type flagellum-dependent cell motility. The configuration of the flagellum p-ring (Flgl) is an I-shaped ring formed around the rod body, whose function may be to protect the motor/substrate
body from shear forces during rotation. Flagellum biosynthetic protein (FlhA) is an important part of the rod structure of flagellum, which, together with FliI and FliH, constitutes the output device of flagellum protein. Experiments showed that flagellins expression was up-regulated with addition of berberine. For example, the expression of the Experimental group protein P40729 was roughly 11 fold than that of the control group. About this point, we suspect that S. Typhimurium flagella are damaged due to berberine. Meanwhile, S. Typhimurium bacteria make more flagellin proteins in order to maintain normal physiological activity as a result of feedback regulation, even though these proteins don't have the potential to form flagella. While this phenomenon does not kill the S. Typhimurium directly, it does help the body to gobble up, lyse, and remove the bacteria during actual treatment.

4. Conclusion

In this study, we profile the proteome of Salmonella enterica serovar Typhimurium under stress of berberine, a alkaloid extracted from plants such as Phellodendron amurense Rupr and Coptis chinensis. We investigated the dose dependent growth inhibition of berberine on S. Typhimurium and chose a proper concentration of berberine for proteomic research. High throughput proteomic profile reveals the target pathway of Salmonella including metabolism enzymes, ribosome proteins and flagellar proteins. The berberine interrupt these proteins regulation and synthesis which result in growth defect. What’s more, the underlying molecular mechanism remain to be elicited.

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