Quantitative proteomics analysis of *Mycoplasma pneumoniae* identifies potential macrolide resistance determinants

shaoli li
Capital institute of pediatric
guanhua xue
Capital Institute of Pediatrics
hanqing zhao
Capital Institute of Pediatrics
yanling feng
Capital Institute of Pediatrics
chao yan
Capital Institute of Pediatrics
jinghua cui
Capital Institute of Pediatrics
xianghui xie
Capital Institute of Pediatrics
jing yuan (yuanjing6216@163.com)
Capital Institute of Pediatrics

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Abstract

Mycoplasma pneumoniae is one of the leading causes of community-acquired pneumonia in children and adolescents. Because of the wide application of macrolides in clinical treatment, macrolide-resistant *M. pneumoniae* strains have become increasingly common worldwide. However, the molecular mechanisms underlying drug resistance in *M. pneumoniae* are poorly understood. In the present work, we analyzed the whole proteomes of macrolide-sensitive and macrolide-resistant strains of *M. pneumoniae* using a tandem mass tag-labeling quantitative proteomic technique, Data are available via ProteomeXchange with identifier PXD022220. In total, 165 differentially expressed proteins were identified, of which 80 were upregulated and 85 were downregulated in the drug-resistant strain compared with the sensitive strain. Functional analysis revealed that these proteins were predominantly involved in protein and peptide biosynthesis processes, the ribosome, and transmembrane transporter activity, which implicates them in the mechanism(s) of resistance of *M. pneumoniae* to macrolides. Our results provide new insights into drug resistance in *M. pneumoniae* and identify potential targets for further studies on resistance mechanisms in this bacterium.

Key Points

1. Macrolide-resistant *M. pneumoniae* infections are very common worldwide.
2. Quantitative proteomic analysis of macrolide resistance of in *M. pneumoniae.*

Introduction

Mycoplasma pneumoniae causes community-acquired pneumonia in children and adolescents (Saraya 2016). Outbreaks of *M. pneumoniae* infections occur every 3–7 years, and 50–80% of individuals in schools and other semi-enclosed spaces are affected by them. With its ability to survive independently *in vitro* and with no cell wall, *M. pneumoniae*, a small prokaryotic bacterium, is naturally resistant to drugs that act on cell walls (Waites et al. 2017). Antibiotics that affect the synthesis of bacterial DNA and protein, such as macrolides, quinolones, and tetracycline, can be used to treat *M. pneumoniae* infections. However, tetracycline can cause tooth yellowing, enamel underdevelopment, gastrointestinal tract stimulant reactions, liver toxicity and other side effects, contraindicating its use for children under 8 years of age. Quinolones also cannot be used in children because they can damage cartilage and joints. Therefore, macrolides are currently the first choice treatments for *M. pneumoniae* infections in children (Lee et al. 2018).

Unfortunately, the widespread clinical application of macrolides has triggered microbial resistance to these agents from the 1970s onwards and, since 2000, macrolide-resistant *M. pneumoniae* strains have become increasingly common in many countries, with drug resistance rates reaching 100% in some areas, thereby posing a significant threat to human health (Tanaka et al. 2017; Cao et al. 2017). Previous studies on drug resistance in *M. pneumoniae* have focused on point mutations in the 23S ribosomal gene and L4 and L22 ribosomal proteins, but whether or not changes at the protein level contribute to

macrolide resistance awaits investigation (Pereyre et al. 2016; Yang et al. 2017). Here, we used a tandem mass tag (TMT)-labeling-based quantitative proteomic technique to identify differentially expressed proteins (DEPs) in macrolide-sensitive versus macrolide-resistant *M. pneumoniae*.

Materials And Methods

Chemicals

RIPA Lysis and Extraction Buffer, a TMT10plex Isobaric Label Reagent Set, and a Pierce™ BCA Protein Assay Kit were purchased from Thermo Fisher Scientific. Urea, triethylammonium bicarbonate (TEAB) buffer (1.0 M, pH 8.5 ± 0.1), Tris (2-carboxyethyl) phosphine (TCEP) hydrochloride solution (0.5 M, pH 7.0), iodoacetamide (IAA), formic acid (FA), acetonitrile (ACN), and methanol were purchased from Sigma (St. Louis, MO, USA). Trypsin from bovine pancreas was purchased from Promega (Madison, WI, USA). Ultrapure water was prepared using a Millipore purification system (Billerica, MA, USA).

Strains

Macrolide-resistant *M. pneumoniae* strain C267 (GenBank No. CP014267), which was isolated in Beijing, China (Li et al. 2016) and the macrolide-sensitive M129 reference strain (ATCC29342) were used in this study. The minimum inhibitory concentration (MIC) values for these strains were determined according to the Clinical & Laboratory Standards Institute document M43A, and the determination was repeated three times for each strain.

Protein extraction and digestion

Proteins were extracted using RIPA Lysis and Extraction Buffer. Protein concentrations were measured using the Pierce BCA Protein Assay Kit. Protein (100 μg) was diluted with 100 mM TEAB to a final volume of 100 μL. TCEP (10 mM) was added to each sample tube and the mixtures were reacted at 56 °C for 1 h. Proteins were alkylated using 20 mM IAA at room temperature in the dark for 1 h. Pre-chilled acetone (−20 °C, 180 μL) was added and the mixture was stored at −20 °C overnight. Samples were centrifuged at 8,000 × *g* for 10 min at 4 °C. The acetone was carefully removed without disturbing the white pellet and the pellet was allowed to dry for 2–3 min. The acetone-precipitated protein pellet (100 μg) was resuspended in 100 μL of 50 mM TEAB. Free trypsin (2 μg) was added to the protein solution and the solution was incubated at 37 °C overnight. Each experiment was repeated three times.

Labeling and peptide fractionation

Immediately before use, the TMT labeling reagents were equilibrated to room temperature. Anhydrous ACN (41 μL) was added to each tube and the reagent was allowed to dissolve for 5 min with occasional vortexing. The samples were labeled with the TMT reagent. The reaction was incubated for 1 h at room temperature. Hydroxylamine (5%, 8 μL) was added to each sample, and the reactions were quenched over a 15 min period. Samples were combined in equal amounts in fresh microcentrifuge tubes, and the mixed

samples were divided into eight fractions using the Pierce™ High pH Reversed-Phase Peptide Fractionation Kit.

LC-MS/MS analysis and database searching

LC-MS/MS analysis was carried out using a Dionex Ultimate 3000 Nano LC system coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA) equipped with an electrospray ionization nanospray source. Mobile phases A and B were 0.1% FA in water and ACN, respectively. The total flow rate was 600 nL/min and a 120-min gradient was set as follows: from 4% to 10% B for 5 min, from 10% to 22% B for 80 min, from 22% to 40% B for 25 min, from 40% to 95% B for 5 min, and held at 95% B for 5 min. The spray voltage was set at 2.0 kV. All MS and MS/MS spectra were acquired in data-dependent acquisition mode and the full mass scan was acquired from *m/z* 300 to 1400 with resolution of 70000.

The raw MS files (The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022220 and 10.6019/PXD022220) were analyzed and searched against the UniProt *M. pneumoniae* database using Proteome Discover 2.1 (Thermo Fisher Scientific). Trypsin was selected as the enzyme and up to two missed cleavages were allowed. Cysteine residue alkylation was set as the static modification, and methionine oxidation was set as the variable modification. The mass tolerance of the precursor was 15 ppm and the peptide false discovery rate was controlled at ≤1%

Bioinformatics analysis

We investigated the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the DEPs using the online OmicsBean resource (http://www. omicsbean.cn/).

Parallel reaction monitoring analysis

The protein expression levels obtained from the TMT analysis were confirmed by quantifying the expression levels of five selected proteins using Parallel Reaction Monitoring (PRM) analysis. Unique peptides from the target proteins were defined according to the TMT data. The proteins (50 μg) were prepared and digested following the TMT analysis protocol. The obtained peptide mixtures were analyzed by nano LC-PRM MS using easy nano-LC (Thermo Fisher Scientific) coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific). The raw data were processed using Skyline 2.6, with the cut-off value set to 0.99. The five product ions with the highest signal intensities were allowed to enter each peptide segment for analysis. Each peptide segment was manually integrated, and the results were exported for data analysis.

Statistical analysis

Statistical analysis was performed using SPSS Statistics software v22.0. Differences in the expression levels of six selected DEPs in the PRM analysis between the macrolide-resistant *M. pneumoniae* strain

C267 and the macrolide-sensitive *M. pneumoniae* strain M129 were determined using a *t*-test, and $p < 0.10$ was taken to indicate statistical significance.

Results

Characteristics of the macrolide-resistant *M. pneumoniae* strain

The macrolide-resistant *M. pneumoniae* strain C267 displayed high MIC values for erythromycin (512 g/mL) and azithromycin (512 g/mL), whereas the values for the reference (sensitive) strain M129 were <0.05 g/mL. The complete genome sequence from strain C267 has been deposited in GenBank under accession number CP014267. The chromosomal DNA from C267 is 816,498 bp long (40.0 mol % G+C), and contains 714 coding sequences, one rRNA operon, and 37 tRNAs. Altogether, 352 single nucleotide polymorphisms (SNPs) were identified between strain C267 and strain M129.

Identification of DEPs

TMT-labeled quantitative proteomes were determined for *M. pneumoniae* strains C267 and M129. Altogether, 7,263 peptides corresponding to 531 proteins were detected. To ensure the reliability of the identification results, we performed peptide length and peptide matching error distribution analyses. Most of the identified peptides were 8–30 amino acids long, and therefore suitable for mass spectrometry (Figure S1A). The mass error rate for the peptides was ±20 ppm, which confirmed that the identification was accurate (Figure S1B).

A strict comparison at fold-change ≥1.2 (for upregulation) or ≤1/1.2 (for downregulation) and a *p*-value cutoff of ≤0.05 was applied to identify the DEPs. When comparing macrolide-resistant strain (C267) with macrolide-sensitive strain (M129), 165 DEPs were observed, with 80 upregulated and 85 downregulated (Supplementary Table S1). As shown in Figure1A and B, a volcano plot and heatmap were employed to analyze the DEPs.

Functional categorization of DEPs

A broad overview of the main differences between the two strains was obtained in the GO and KEGG analyses. We obtained the top 10 significant GO terms for DEPs in the following categories: biological process (BP), cellular component (CC), and molecular function (MF) (Figure 2). Translation, cellular protein metabolic process, peptide biosynthetic process, peptide metabolic process, amide biosynthetic process, cellular amide metabolic process, cellular macromolecule biosynthetic process, and macromolecule biosynthetic process were the most significantly enriched in the BP category. In the CC category, cytoplasmic part, ribosome, intracellular ribonucleoprotein complex, ribonucleoprotein complex, and macromolecular complex were found to be significantly enriched. Notably, proton-transporting ATP synthase complex, coupling factor F(o), proton-transporting two-sector ATPase complex, and proton-transporting domain, were among the top 10 significantly enriched CC terms. In the MF category, the following DEPs were highly enriched: structural molecule activity, structural constituent of ribosome,

monovalent inorganic cation transmembrane transporter activity, and inorganic cation transmembrane transporter activity terms.

Thirty-two KEGG pathways were annotated for the 165 DEPs (Table 1). Ribosome was the most significant and abundant pathway. Twenty-eight DEPs were involved in the ribosome pathway, including RPSZ, RPLW, RPSB, RPSC, RPLL, RPSH, RPLO, RPLU, RPLJ, RPLK, RPLF, RPSD, RPLD, RPLA, RPMC, RPMB, RPLN, RPLP, RPLV, RPLM, RPST, RPLC, RPLE, RPLX, RPSG, RPME, RPLQ and RPMA. ECFA2, ECFA1, MPN_611, PSTA, POTC, POTA and MPN_058 are associated with ABC transporters. A protein interaction network was constructed for the DEPs (Figure 3), and the interactions we identified in it provide important information about their functions and behaviors, thereby providing a useful pointer for understanding the macrolide resistance mechanisms of *M. pneumoniae*.

Table 1. KEGG annotation of DEPs between the macrolide-resistant *M. pneumoniae* C267 strain and the macrolide-sensitive M129 strain.

Validation of DEPs

Because antibodies suitable for use in *M. pneumoniae* are rare, targeted, quantitative MS approaches such as PRM and multireaction monitoring are essential for DEP confirmation. To validate the results obtained from TMT-based proteomics, we examined the expression levels of several candidate proteins by PRM. Because this technique requires the signature peptide of the target protein to be unique, we selected six proteins with unique signature peptide sequences for PRM analysis. The fold-changes for these proteins differed significantly between the macrolide-resistant C267 strain and the macrolide-sensitive M129 strain at $p < 0.05$, a result in agreement with the findings from the TMT analysis (Table 2).

Table 2. Confirmation of the DEPs detected in the TMT analysis using PRM analysis.

Discussion

M. pneumoniae is one of the main pathogens to cause community-acquired respiratory tract infections and, because these infections can lead to bronchitis and atypical pneumonia as well as a variety of extrapulmonary complications, this pathogen can seriously endanger the health of children and adolescents (Uldum et al. 2012; Principi et al. 2013; Waites et al. 2004). Because it lacks a cell wall, *M. pneumoniae* is resistant to β-lactams and other antibiotics that act on bacterial cell walls, but it is (in principle) sensitive to macrolides, tetracyclines, and quinolones, because these agents inhibit or affect the synthesis of bacterial proteins and nucleic acids. However, the increasing prevalence of macrolide-resistant *M. pneumoniae* is a significant problem because clinical treatments depend on macrolide antibiotics (Suzuki et al. 2013; Peuchant et al. 2009; Wolff et al. 2008; Dumke et al. 2010; Bajantri et al. 2018). Moreover, some studies have indicated that patients infected with macrolide-resistant strains have greater clinical manifestations and longer disease durations than those infected with wild-type (sensitive)

strains (Zhou et al. 2014; Liu et al. 2010). Therefore, research into the drug resistance mechanism(s) of *M. pneumoniae* and implementing rational clinical drug use is now an urgent priority.

Macrolides bind to ribosomal subunits from bacteria and inhibit protein synthesis by blocking peptide transfer and mRNA displacement (Roberts 2004; Giedraitienė et al. 2011). Previous studies on *M. pneumoniae* resistance focused on point mutations in the 23S ribosomal gene and ribosomal proteins L4 and L22 (Principi et al. 2013; Matsuoka et al. 2004; Suzuki et al. 2006). Our previous study confirmed that macrolide-resistant strain C267 harbors an A to G mutation at nucleotide position 2,063 within domain V of the 23S rRNA gene (Li et al. 2017). In the present study, the GO analysis of DEPs showed that translation, peptide biosynthetic processes, ribosome, intracellular ribonucleoprotein complex, ribonucleoprotein complex, and the structural constituents of ribosomes were significantly enriched terms in the macrolide-resistant strain when compared with the sensitive strain (Figure 3). The KEGG analysis also revealed that 28 DEPs were involved in ribosomal pathways (Table 1). Notably, all the ribosomal proteins were downregulated in the resistant strain unlike those in the sensitive strain (Figure 4). Saito et al. (1969) found that erythromycin–ribosome complex formation decreased in erythromycin-resistant *Staphylococcus aureus* strains (Saito et al. 1969). The reduced ability of ribosomes from resistant cells to bind erythromycin and other macrolide antibiotics has been used to demonstrate induced resistance, as has the increased resistance of these ribosomes to inhibition by macrolide antibiotics in cell-free protein synthesis (Shimizu et al. 1970; Weisblum et al. 1971; Allen 1977). Therefore, ribosomal proteins play an important role in drug resistance in *M. pneumoniae*. However, the exact mechanism(s) underpinning the involvement of ribosomal proteins in drug resistance need(s) to be investigated further.

Transporters are another important type of protein involved in drug resistance. Transporters pump a drug out of the cell or the cellular membrane, thereby keeping the intercellular concentrations low (Roberts 2004). An active efflux system, possibly an ABC-type efflux pump, was suggested to be involved in resistance to ciprofloxacin in wall-less *M. hominis* (Raherison et al. 2002). The existence of an active efflux process in *M. hominis* was also implicated in resistance to erythromycin because an ABC transporter inhibitor was able to increase erythromycin uptake levels by more than two-fold (Pereyre et al. 2002). Our previous study showed that a macrolide efflux pump, possibly an ABC-type efflux pump, may contribute to macrolide resistance in *M. pneumoniae* C267 (Li et al. 2017). The proteomics results from the present study support this assumption. KEGG analysis of the DEPs between the macrolide-resistant strain C267 and macrolide-sensitive strain M129 indicates that the following seven DEPs are associated with ABC transporters: ECFA2, ECFA1, MPN_611, PSTA, POTC, POTA and MPN_058. In addition, spermidine transmembrane transporter activity, monovalent inorganic cation transmembrane transporter activity, and inorganic cation transmembrane transporter activity were among the 10 most significantly enriched MF terms, suggesting that transporters are significant players in macrolide resistance in *M. pneumoniae*.

Cell membranes consist of a lipid bilayer in which proteins that have important cellular functions, such as receptors, transporters, and enzymes, are embedded (Spector et al. 1985). The cell membranes play an important role by acting as a permeability barrier to the entry of diverse chemical agents (Nikaido 2003).

Alteration of the cell membrane's lipid composition can be related to drug resistance. Changed membrane phospholipid and sterol compositions were observed in both clinical and *in vitro*-adapted azole-resistant *Candida albicans* isolates (Mukhopadhyay et al. 2002; Hitchcock et al. 1986; Kohli et al. 2002; Löffler et al. 2000). It was reported that benzyldimethyltetradecylammonium chloride-adapted *Pseudomonas aeruginosa* cells showed variations in membrane fatty acid composition (Nikaido 2003). In our analysis, two lipid-related pathways, glycerolipid metabolism and glycerophospholipid metabolism, were altered in resistant *M. pneumoniae* C267 compared with sensitive *M. pneumoniae* M129 (Table 1). Such changes are possibly one of the causes of *M. pneumoniae* drug resistance and, as such, they warrant further investigation.

Drug resistance in *M. pneumoniae* is an increasingly serious problem, and further research into the mechanisms underlying it in this bacterium is urgently needed. Our study provides a global analysis of protein expression changes between the macrolide-resistant C267 strain and the macrolide-sensitive M129 strain of *M. pneumoniae*. We identified several important pathways and candidate proteins that are potential targets for further studies on macrolide resistance in *M. pneumoniae*. However, one limitation of this study is that the sample size was too low. Therefore, our future goal is to test more strains to confirm the relationship between protein expression and drug resistance in *M. pneumoniae*.

Declarations

Competing interests

The authors declare that there are no competing interests associated with this manuscript.

Ethics approval and consent to participate

The present study was performed in compliance with the Helsinki Declaration (Ethical Principles for Medical Research Involving Human Subjects) and was approved by the research board of the Ethics Committee of the Capital Institute of Pediatrics, Beijing, China. The patient data were anonymously reported. Based on the guidelines of the Ethics Committee of the Capital Institute of Pediatrics, informed consent was not sought from the patients.

Consent for publication

Not applicable

Availability of data and material

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

Code availability

Not applicable

Authors' contributions

LS and XG conceived and designed the research. ZH and FY conducted the experiments. YC and CJ contributed new reagents or analytical tools. LS and YJ analyzed the data. LS and XX wrote the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1. KEGG annotation of DEPs between the macrolide-resistant *M. pneumoniae* C267 strain and the macrolide-sensitive M129 strain.

Pathway name	Count	DEPs
Ribosome	28	RPSZ RPLW RPSB RPSC RPLL RPSH RPLO RPLU RPLJ RPLK RPLF RPSD RPLD RPLA RPMC RPMB RPLN RPLP RPLV RPLM RPST RPLC RPLE RPLX RPSG RPME RPLQ RPMA
Metabolic pathways	22	DEOD DNAX PLSY GLPK TMK PDHC CSD ULAE ATPF NRDE ATPG MPN_450 RPOE ATPA PYRH NRDF THYA THII ULAF TPIA ATPE NADK
Pyrimidine metabolism	9	DEOD DNAX TMK NRDE MPN_450 RPOE PYRH NRDF THYA
Aminoacyl-tRNA biosynthesis	8	HISS VALS TRPS GLYQS LEUS PHET METG ALAS
ABC transporters	7	ECFA2 ECFA1 MPN_611 PSTA POTC POTA MPN_058
Purine metabolism	6	DEOD DNAX NRDE MPN_450 RPOE NRDF
Biosynthesis of secondary metabolites	5	DEOD PLSY MPN_051 PDHC TPIA
Oxidative phosphorylation	4	ATPF ATPG ATPA ATPE
Homologous recombination	4	DNAX MPN_450 SSB RUVB
DNA replication	4	DNAX DNAB MPN_450 SSB
Microbial metabolism in diverse environments	4	PDHC ULAE ULAF TPIA
Mismatch repair	3	DNAX MPN_450 SSB
Protein export	3	SECG LSPA FTSY
Selenocompound metabolism	2	CSD METG
Glycerolipid metabolism	2	PLSY GLPK
Nicotinate and nicotinamide metabolism	2	DEOD NADK
Ascorbate and aldarate metabolism	2	ULAE ULAF
Glycerophospholipid metabolism	2	PLSY MPN_051

Bacterial secretion system	2	SECG□FTSY
Fructose and mannose metabolism	2	TPIA□MTLD
Glycolysis / Gluconeogenesis	2	PDHC□TPIA
Carbon metabolism	2	PDHC□TPIA
Biosynthesis of antibiotics	2	PDHC□TPIA
Two-component system	1	MPN_611
Sulfur relay system	1	THII
RNA polymerase	1	RPOE
Citrate cycle (TCA cycle)	1	PDHC
Base excision repair	1	MUTM
One carbon pool by folate	1	THYA
RNA degradation	1	RNJ
Pyruvate metabolism	1	PDHC
Biosynthesis of amino acids	1	TPIA

Table 2. Confirmation of the DEPs detected in the TMT analysis using PRM analysis.

Accession no.	Gene Symbol	Fold-change (C267/M129) in PRM	P-value in PRM	Fold-change (C267/M129) in TMT	P-value in TMT
P75295	MPN_491	12.51738715	1.14223E-05	2.268532831	2.22037E-05
P75121	MPN_670	1.766381125	0.028324373	1.266310644	3.47397E-05
P75603	MPN_090	1.654676801	0.021731425	1.231982961	0.000180854
P75392	pdhC	1.549656015	0.009815993	1.240981292	5.22141E-05
P75527	def	1.514331423	0.036288214	1.302137569	0.000302493
P75236	MPN_542	1.37969945	0.098842902	1.493066835	0.000621553

Figures

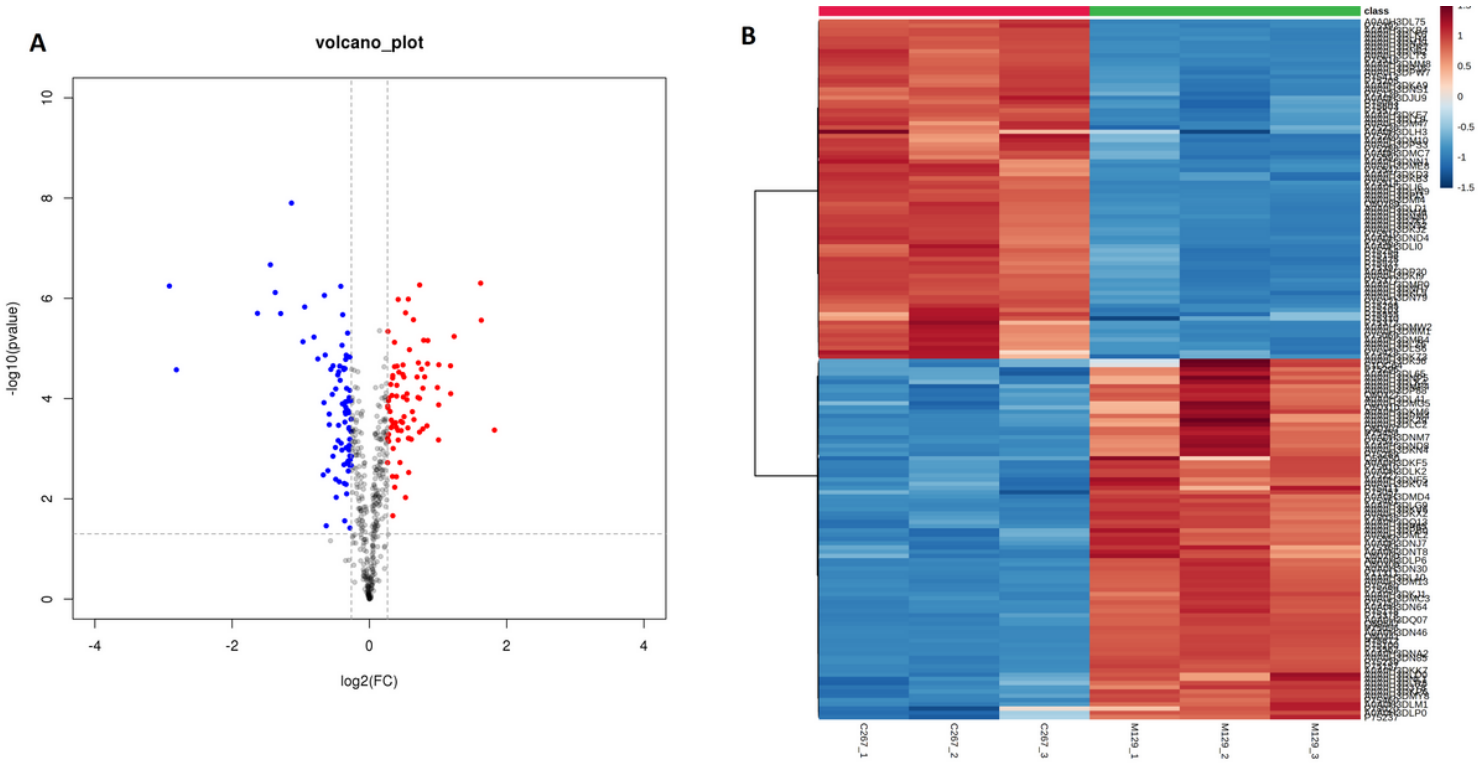

Figure 1

Identification of differentially expressed proteins (DEPs). (A) Volcano plots of the DEPs. The horizontal coordinate indicates the log2(FC) values and the vertical coordinate indicates the -log10 values. (B) Heatmap of DEPs. The expression values shown in different colors denote different protein expression levels.Gene ontology (GO) based on DEPs. Top 10 significant GO terms for the DEPs in each category (sorted by the -log10P−values from high to low). Blue, red and yellow bars represent different GO categories. BP, biological process. CC, cell cellular. MF, molecular function.

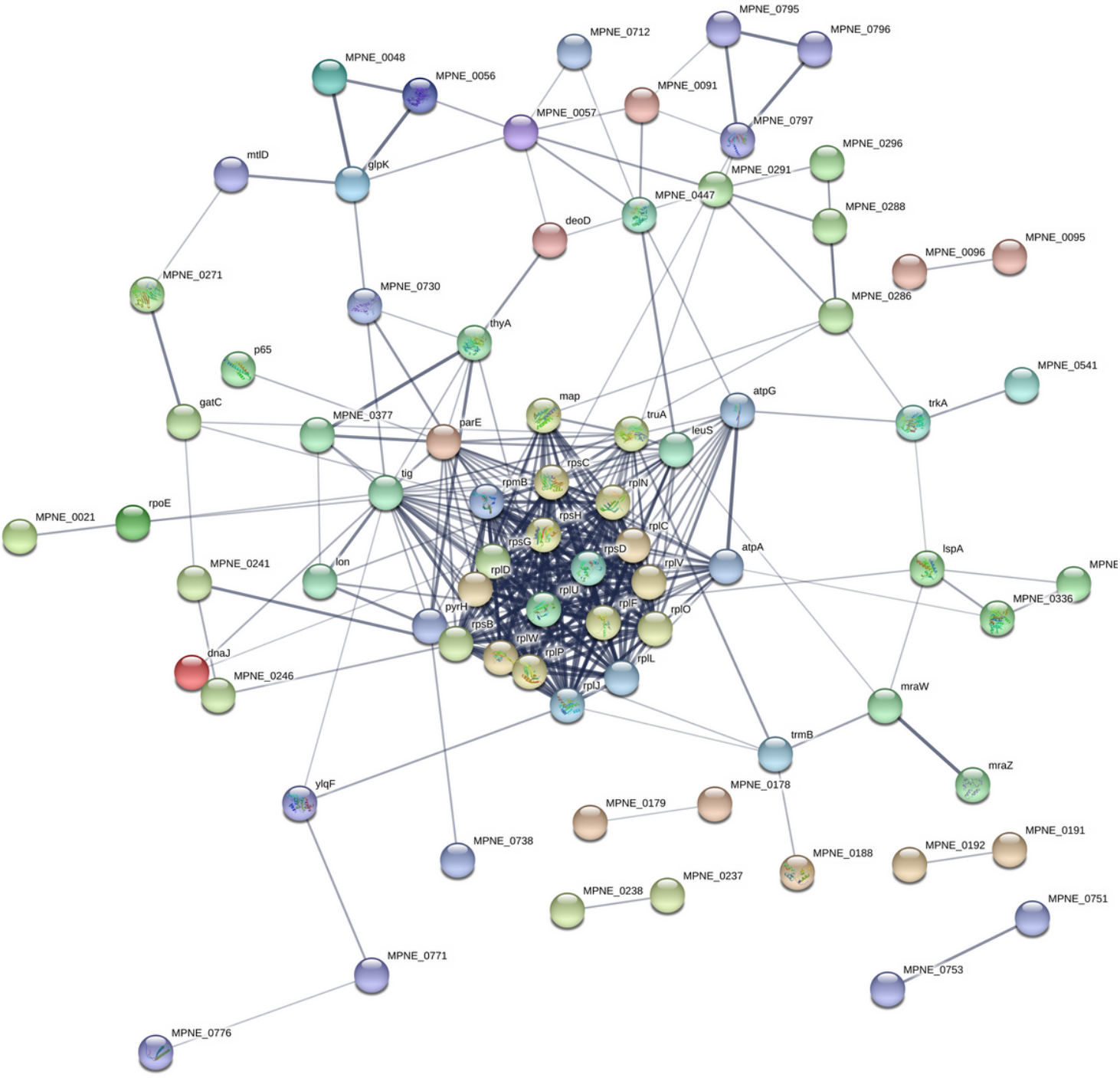

Figure 2

STRING protein network analysis on the proteins identified as being significantly differentiated. Proteins were considered significant at a p-value of <0.05. The thicker the connecting lines in between the proteins the stronger the protein-protein associations.

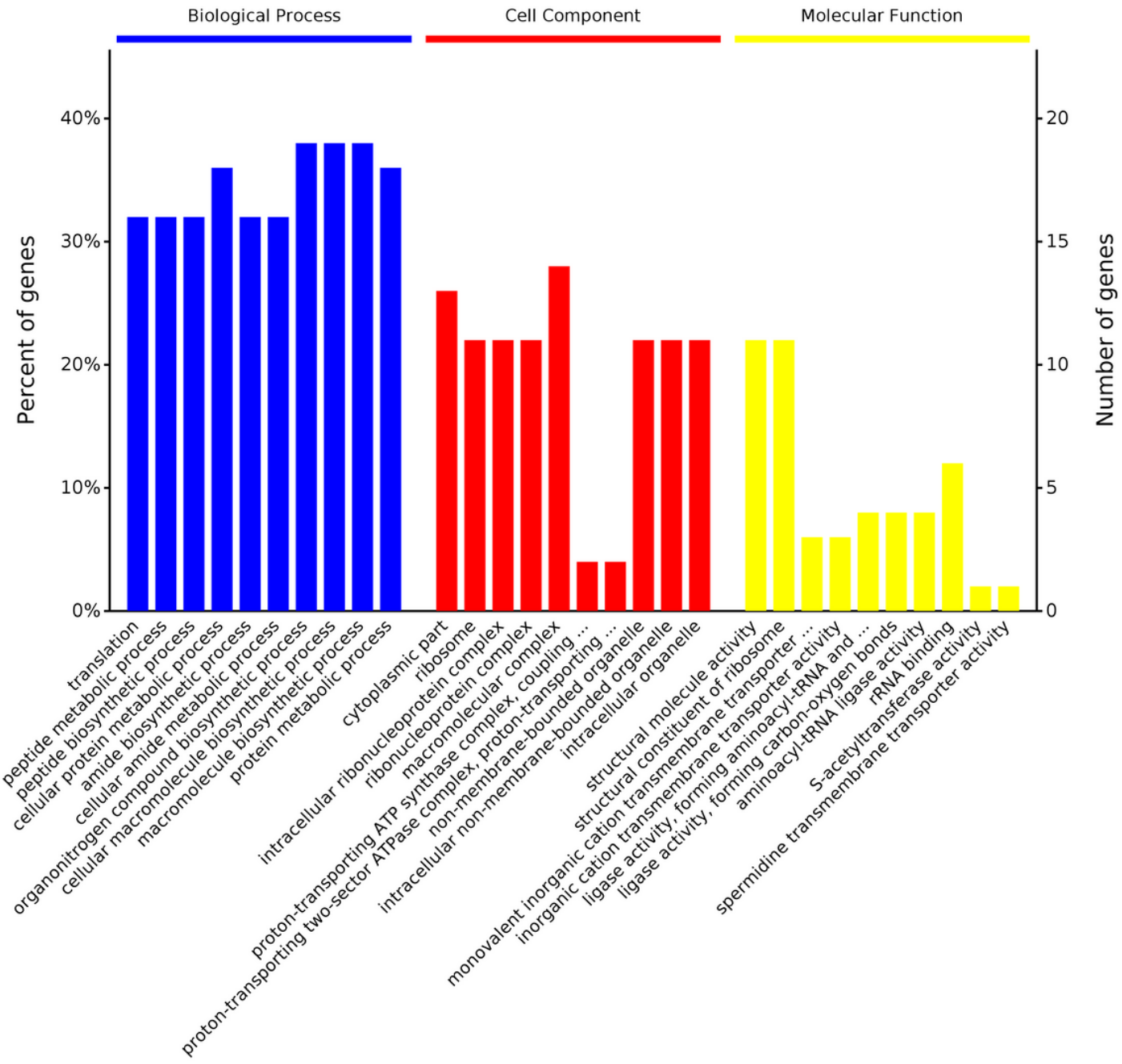

Figure 3

Gene ontology (GO) based on DEPs. Top 10 significant GO terms for the DEPs in each category (sorted by the -log10P−values from high to low). Blue, red and yellow bars represent different GO categories. BP, biological process. CC, cell cellular. MF, molecular function.

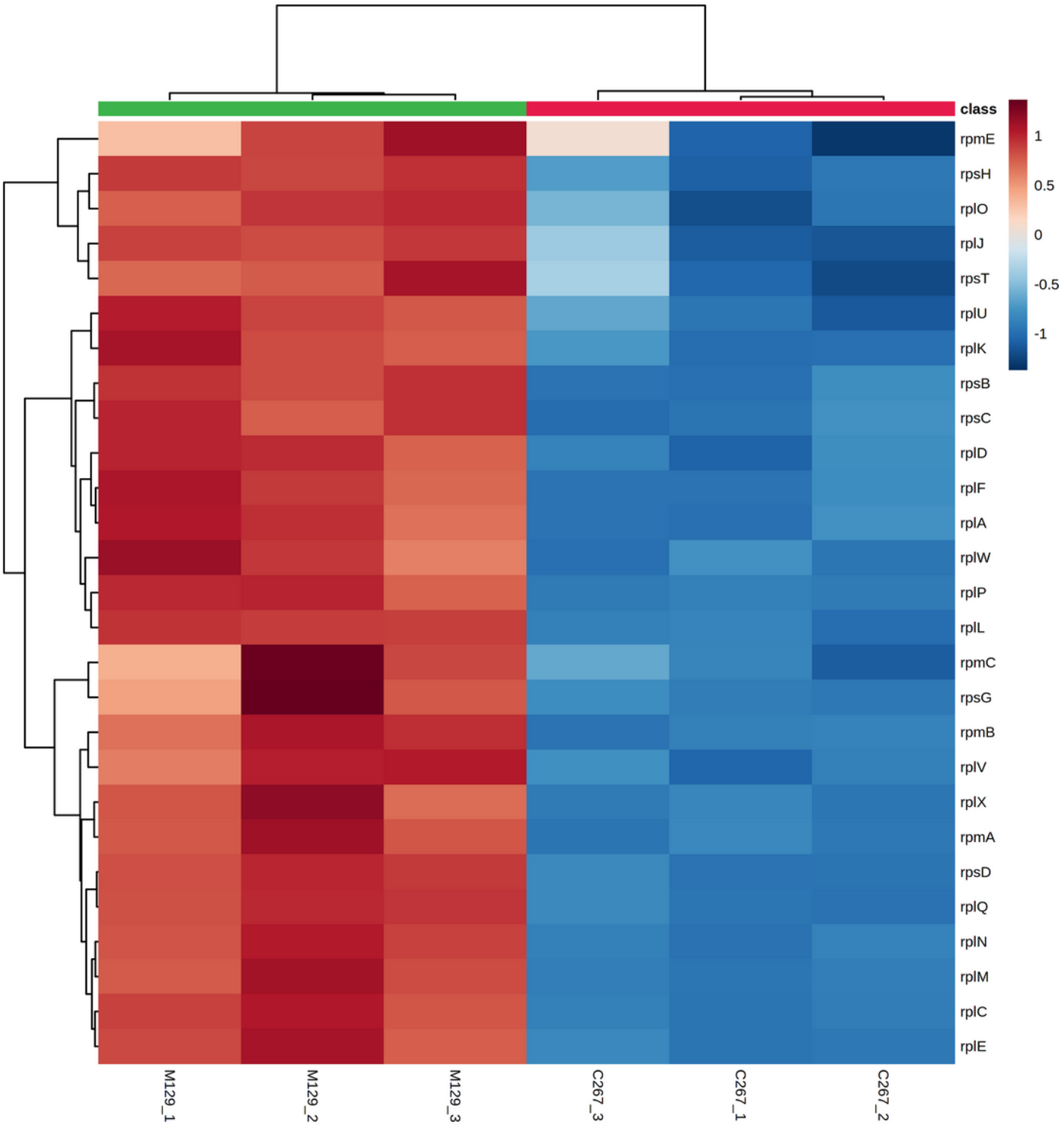

Figure 4

Heatmap of ribosome proteins. The expression values shown in different colors denote the different protein expression levels.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FiguerS1A.png
- FigureS1B.png
- TableS1.pdf