TMT-based proteomic analysis of the antibiotic effects of ShangKeHuangShui against *Straphylococcus aureus*.

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Abstract

Post-traumatic infection is a serious orthopedic trauma complication commonly caused by *Staphylococcus aureus* (SAU). ShangKeHuangShui, also known as Yellow Aqueous Concentrate of Traumatology Herbs (YACTH), is prepared from six Chinese herbal medicines, which has been used for decades in our hospital to prevent post-traumatic infection.

In the present study, we investigated the *in vitro* antibacterial effects and underlying mechanism of YACTH against SAU. YACTH exhibited significant antibacterial activity against SAU with the minimum inhibitory concentration (MIC) of 8.625 mg/mL. Proteomic analysis based on Tandem mass tag (TMT) showed different protein expression levels in SAU under the YACTH and control conditions. Compared to the control group, the expression level of 490 proteins of YACTH treated group significantly changed (>1.2 fold, $P < 0.05$). Biological informatics analysis showed that these differential proteins were widely involved in a variety of biological processes and multiple metabolic pathways. We then selected 26 target proteins to further conduct Parallel reaction monitoring (PRM). The results revealed that the 9 down-regulated proteins were mainly involved in RNA polymerase, oxidative phosphorylation, ABC transporter and glycolysis; whereas, the 17 up-regulated proteins are related to RNA degradation, mismatch repair and ribosome.

In conclusion, YACTH, which contains the water-soluble components of six Chinese herbs, is first reported to exhibit antibacterial activity against SAU *in vitro*. The results provide novel insights into the antibacterial mechanisms of YACTH on protein networks, which may help us find new potential antibiotic targets.

Introduction

Staphylococcus aureus (SAU), one species of coagulase Gram-positive staphylococcus, is widely found in the skin of mammals [1]. Due to its infectiousness and resistance to high temperature [2], SAU has become a major nosocomial pathogenic cause of superficial lesions, systemic infections, and several toxemic syndromes [3]. Skin injuries with open wounds can be easily infected by SAU and result in impetigo and folliculitis [4]. Infection with SAU is a serious complication for orthopedics trauma and is quite common in open fracture management [5].

To date, a variety of antibiotics have been invented to fight against SAU infection. Yellow Aqueous Concentrate of Traumatology Herbs (YACTH) is developed by six Chinese herbal medicines including Coptis Chinensis, Philodendron Chinensis, Gardenia jasminoides, Lithospermum, peppermint, and alum [6]. Its water-soluble components of berberine, geniposide, palmatine, and Phellodendron are potential natural antibacterial compounds that are reported to reduce subside swelling, invigorate blood circulation, promote tissue regeneration and improve wound healing [7,8]. However, whether such improvement was achieved by anti-inflammatory or antibacterial effects remained unknown, as well as the relevant molecular mechanism. In the presence of microorganisms, soft tissue injury and the consequent decline of antibacterial capability are the main causes of infection [9]. Considering YACTH contains antibacterial components, suggested that YACTH may promote wound healing through suppressing bacterial activities. Typically, the single component chemical compound, such as beta-lactam antibiotics inhibit SAU through bifunctional transglycosylase-transpeptidases PBP [10] and tetracyclines that bind to the 30S subunit of the ribosome to interfere with the protein synthesis of SAU [11], solely have one molecular target. Previous studies provided many antibiotics with clear

mechanisms. The majority of those antibiotics suppress SAU through targeting the cell envelope and proteins that are involved in protein synthesis and nucleic acids biosynthesis [12].

YACTH, as a compound formulation containing numbers of active antibacterial components, might have multi-target antibacterial activities, which makes it difficult to study the mechanism of how YACTH inhibits SAU. Proteomics is the analysis of the entire protein complement of a cell, tissue, or organism under a specific, defined set of conditions [13]. Up to date, proteomics could be applied to many fields due to the rapid development of technologies including protein fractionation techniques, mass spectrometry (MS) technology, bioinformatics, etc. The protein expression changes could be detected through MS, and reveal pathway information through informatics [14].

We hypothesized that YACTH inhibits SAU by regulating a variety of biological processes and multiple metabolic pathways. In the present study, we employed proteomics analysis to provide novel insights into the antibacterial mechanisms of YACTH on protein networks and explore some potential antibiotic targets.

Materials and methods

Evaluation of Antibacterial Activity of YACTH

Strain and Culture Condition

The strain *S.aureus* ATCC 25923 used in this study was kept in lab and cultured aerobically in broth medium（Qingdao Hope Bio-Technology Co., Ltd）on a shaking incubator at 250 rpm, 37 ℃.

Antibacterial Activity of YACTH

Antibacterial activity of YACTH was tested according to the agar diffusion method as reported previously with slight modification [15]. The YACTH extracts were filtered through 0.22 μm sterilizing filters. Briefly, 100 μL bacterial suspension containing 10^6 CFU/mL of SAU was evenly smeared on Mueller-Hinton ager plates. Oxford cups (outside diameter of about 8.0 mm) were placed on the plates.After 20 min, 80 μL of YACTH were added to the Oxford cups. Saline was used in parallel experiments as a negative control. The diameters of the inhibitory zones (DIZs) were measured after incubation of the plates at 37 °C for 24 h, which were expressed in millimeter (mm) and recorded as mean ± standard deviation (SD). DIZ values less than 8 mm were considered as no inhibition zone (NIZ). All experiments were performed in triplicate.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) were determined by a micro-dilution test according to the method described by Jeong [16]. Studies were conducted according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) [17]. Two-fold serial dilutions of YACTH were prepared in sterile broth media ranging from 0.54-34.5 mg/mL. Afterwards, 180 μL of diluted YACTH was mixed with 20 μL bacterial suspension (approximately 10^7 CFU/mL) in the 96-well plates. The negative control contained 180 μL diluted YACTH with 20 μL 0.9% NaCl. The plates were incubated at 37 ºC for 24 h. The MIC was defined as the concentration at which the corresponding well showed no visible bacterial growth after incubation at 37 ºC for 24 h. Afterwards, a subculture of 50 μL from each well with no visible bacterial growth was directly

incubated onto the nutrient agar plate incubating at 37 ºC for 24 h, and the lowest on centration at which there was no colony growth was defined as MBC.

Biochemistry experiments

Protein extraction and digestion

The proteins were lysed and extracted with SDT buffer (4% SDS,100 mM Tris-HCl,1 mM DTT, pH 7.6), and then quantified with the BCA protein assay kit (Bio-Rad, USA). After trypsin digestion, the peptides of each sample were desalted and concentrated by vacuum centrifugation and reconstituted in formic acid (40,0.1% (v/v)).

Filter-aided sample preparation (FASP Digestion)

200 μg of proteins of each sample were treated with 30 μL SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0). Used UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) to remove detergent, DTT, and other low-molecular-weight components by repeated ultrafiltration (Microcon units, 10 kD). Added 100 μL iodoacetamide (100 mM IAA in UA buffer) to block reduced cysteine residues. Incubated the samples for 30 min in a dark environment. Washed the filters with 100 μL UA buffer three times and then 100 μL 25mM NH_4HCO_3 buffer twice. Digested protein suspensions with 4 μg trypsin (Promega) in 40 μL 25 mM NH_4HCO_3 buffer at 37 °C overnight. The resulting peptides were collected as a filtrate. The peptides of each sample were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma), concentrated by vacuum centrifugation and

reconstituted in 40 µL of 0.1% (v/v) formic acid. Estimated the peptide content by UV light spectral density at 280 nm（extinctions coefficient= 1.1, 0.1% (g/L)）.

SDS-PAGE

Mixed 20 µg of protein for each sample with loading buffer (5X) and boiled it for 5 min. The proteins were separated on 12.5% SDS-PAGE gel (constant current 14 mA, 90 min), and stained by Coomassie Blue R-250.

TMT Labeling

Peptides (100 µg) of each sample were labeled with TMT reagent according to the manufacturer's instructions (Thermo Scientific).

High pH Reversed-Phase Fractionation

Labeled peptides were fractionated by High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific). The dried peptides were reconstituted and acidified with 0.1% TFA solution and loaded on the equilibrated, high-pH, reversed-phase fractionation spin column. Peptides were attached to the hydrophobic resin under aqueous conditions and desalted by water through low-speed centrifugation. Collected 10 fractions from the gradient elution of the attached peptides by increasing acetonitrile concentrations under high-pH. Desalted the collected fractions on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma) and concentrated them by vacuum centrifugation.

LC-MS/MS analysis

Performed LC-MS/MS analysis on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 60 min. At a flow rate of 300 nL/min, the peptides were applied to a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm*2 cm, nanoViper C18) connected to the C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% Formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid). The mass spectrometer was operated in positive ion mode. MS data were acquired via the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation using a data-dependent top10 method dynamically. Set automatic gain control (AGC) target to 3e6 and maximum inject time to 10 ms with a dynamic exclusion duration of 40.0 s. Acquired survey scans at a resolution of 70,000 at m/z 200 and set resolution for HCD spectra to 17,500 at m/z 200, and isolation width was 2 m/z. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. Ran the instrument with peptide recognition mode enabled.

Identification and quantitation of proteins

Searcher the MS raw data for each sample using the MASCOT engine, Uniprot (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 software for identification and quantitation analysis.

Bioinformatic analysis

Cluster analysis

Performed hierarchical clustering analysis through Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and Java Treeview software (http://jtreeview.sourceforge.net) and selected Euclidean distance algorithm for similarity measure and average linkage clustering algorithm (clustering uses the centroids of the observations) for clustering.

Subcellular localization

A multi-class SVM classification system, CELLO (http://cello.life.nctu.edu.tw/), was used to predict protein subcellular localization.

Domain annotation

Search protein sequences via the InterProScan software. Identified protein domain signatures from the InterPro member database P fam.

GO annotation

Locally searched the protein sequences of the selected differentially expressed proteins using the NCBI BLAST+client software (NCBI-blast-2.2.28 ± win32.exe) and found homologue sequences on InterProScan. Mapped then gene ontology (GO) terms and annotated the sequences using the software program Blast2GO. Plotted the GO annotation results by R scripts.

KEGG annotation

Blasted the studied proteins against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/) to retrieve their KEGG orthology identifications. Subsequently mapped the matched proteins to pathways in KEGG.

Enrichment analysis

The whole quantified proteins were considered as background dataset. The enrichment analysis was applied based on Fisher's exact test. Further applied Benjamini- Hochberg correction for multiple testing to adjust derived p-values. Only considered functional categories and pathways with p-values under a threshold of 0.05 as significant.

Statistical Analysis

Statistical analysis was conducted with two-tailed t-test. The P value was set at < 0.05. Fisher's exact test was used to calculate the P value compare the distribution of each GO classification or KEGG pathway in the target protein set and the total protein set, and the enrichment analysis of GO annotation or KEGG pathway annotation was performed on the target protein set.

Results

Antibacterial Activity of YACTH

The antibacterial activity of YACTH against SAU was measured by Oxford cup and

micro-dilution methods, diameters of inhibition zone (DIZ) were appreciated as follows: not sensitive (diameter ≤ 8.0 mm), moderately sensitive (8.0 < diameter ≤ 14.0 mm), sensitive (14.0 < diameter ≤ 20.0 mm), and extremely sensitive (diameter >20.0 mm) [15]. The mean of DIZ values was 28.04mm (± 0.94 mm), and the corresponding MIC and MBC against SAU were 8.625 mg/mL and 17.25 mg/mL, respectively.

LC-MS/MS Analysis

In this study, the TMT technique was applied to analyze the proteomics of SAU treated with YACTH. The experimental procedure is illustrated in Fig 1.

Fig 1. Experimental procedure of LC-MS/MS analysis.

Fig 1 shows the experimental procedures of our study. The YACTH group was compared to the control group. Each group contained three samples and went through protein preparation, labeling, fractionation, LC-MS/MS, protein identification and quantification, and bioinformatic analysis.

In LC-MS/MS analysis, a total of 470327 products were detected on the spectrum with 63749 matched with recorded data. Among these fractionated products, a total of 23544 peptides were identified and 16490 were recognized as unique, while 2583 proteins were quantified out of the 2605 identified proteins. Compared to the control group, the expression of 490 proteins was significantly affected by the treatment of YACTH ($P < 0.05$), while other proteins remained to be unchanged. Among these proteins, 241 proteins were up-regulated and 249 proteins were down-regulated (Fig 2A). To confirm the regulatory effects of YACTH on the growth and metabolism of SAU, a cluster plot was constructed to demonstrate the significant difference in protein expression between the control group and the YACTH treatment group (Fig 2B).

Fig 2. Volcano plot and heat map of SAU protein expressions under YACTH treatment. Fig 2A is the volcano plot of the proteins that were affected by the YACTH treatment. While the grey dots represent the proteins that remained unchanged, the red dots represent the upregulated proteins and the blue dots represent the down-regulated proteins in the presence of YACTH. Fig 2B is the heat map that depicts the proteins being influenced by YACTH treatment. Each column represents a group of proteins. The protein expression levels are normalized by log2 and labeled in different colors. The up-regulated proteins are colored in red, and down-regulated proteins are colored in blue.

Functional Analysis

In sub-cellular localization analysis, 384 cytoplasmic proteins, 83 membrane proteins, and 87 extracellular proteins were identified (S1 Fig). In addition, we conducted domain analysis to match YACTH regulated proteins with acknowledged structural domains. The majority of structural domains could be found in the proteins affected by YACTH treatment (Fig 3A). Typical structural domains such as ABC transporter, LPXTG cell wall anchor motif, YSIRK type signal peptide, radical SAM superfamily, and B domain, etc were observed. To study the overall response of SAU in the presence of YACTH, we performed GO analysis to assign the individual protein expression changes into certain groups. Take the example of the Top 20 enriched Go terms, proteins with significant expression changes were grouped into six biological processes with the highest number of proteins belongs to the cellular amide metabolic process, eight molecular functions with the highest number of proteins belong to RNA binding, six cellular components with the number of proteins almost evenly distributed in each cellular component (Fig 3B).

Fig 3. Domain analysis and GO analysis of the SAU proteins that were affected by the YACTH treatment.

Fig 3A shows the top 20 proteins that were matched with structural domains from the protein data bank. The length of the blue bars represents the number of proteins. Fig 3B depicts the GO enrichment analysis of the top 20 proteins. The proteins were categorized by biological process (BP), molecular function (MF), and cellular component (CC). The p-value is represented by the color intensity. The more intense red color represents a lower p-value.

In addition, we performed KEGG pathway analysis, which indicated that YACTH treatment regulated the expression of abundant SAU proteins, which were widely involved in a variety of biological processes and multiple metabolic pathways (Fig 4). The size of the dots represents the number of changed proteins, and the biggest protein number belongs to the ribosome, which is the key organelle responsible for translational activities. Other vital pathways related to SAU growth and metabolism, such as RNA polymerase, aminoacyl-tRNA biosynthesis, pyruvate metabolism, and aminoacyl-tRNA biosynthesis, were also presented in our data (Fig 4).

Fig 4. KEGG Pathway analysis of the SAU proteins that were affected by the YACTH treatment.

The proteins influenced by the YACTH treatment are assigned to different pathways, while the top 20 pathways are exhibited in Fig 4. Five grades (from 1 to 5) of the p-value, converted by –log10, were presented in colors while the size of the dots represents the number of proteins.

PRM analysis

According to the GO and KEGG analysis of TMT results, 26 target proteins were selected to conduct PRM analysis; 6 of these proteins were significantly down-regulated and 20 proteins up-regulated as shown in Table 1. The up-regulated and down-regulated trends of all proteins of TMT were consistent with that of PRM

309 analysis. For example, the DEAD-box RNA helicase cshA related to RNA
310 degradation was up-regulated by 1.25-fold and 1.35-fold, while Putative DNA-
311 directed RNA polymerase subunit delta related to RNA polymerase was down-
312 regulated to 0.36 times and 0.07 times in TMT and PRM analysis, respectively. The
313 up-regulated proteins were primarily involved in the pathways related to the
314 ribosome, mismatch repair, aminoacyl-tRNA biosynthesis, and ABC transporter, etc.
315 Three proteins associated with Mismatch repair were up-regulated 1.25-1.27 times.
316 Six proteins in the ribosome, two proteins in ABC transporter, and one protein in
317 glycolysis were also up-regulated. On other hand, the down-regulated proteins were
318 mainly involved in the pathways related to RNA polymerase and the two-component
319 system. At the same time, some pathways were regulated in both up-and down-
320 directions. In glycolysis, three proteins were upregulated, while two proteins were
321 down-regulated; in ABC transporter, two proteins were up-regulated, while three
322 proteins were down-regulated. All the TMT results are statistically significant, and
323 PRM analysis further confirmed the results of TMT (Table 1).

Table 1 PRM analysis of SAU proteins that were affected by the YACTH treatment.

Map Name	Protein Name		Gene Name	TMT		PRM
				Ratio YACTH/Control	TTEST YACTH/Control	Ratio YACTH/Control
Mismatch repair	A0A0E0VPG2	RecJ	ST398NM01_1699	1.27	0.027	1.46
	Q6GFF3	DNA ligase	ligA	1.25	0.025	1.29
	P64319	ATP-dependent DNA helicase PcrA	pcrA	1.25	0.01	1.33
RNA degradation	A0A077UMU7	DEAD-box ATP-dependent RNA helicase CshA	cshA	1.25	0.049	1.35
RNA polymerase	P66725	DNA-directed RNA polymerase subunit omega	rpoZ	0.67	0.045	0.23
	A0A5S9C4T9	Putative DNA-directed RNA polymerase subunit delta	rpoE	0.36	0.004	0.07
Ribosome	A7X5D9	50S ribosomal protein L18	rplR	1.81	0.016	1.94
	A0A033UZC9	30S ribosomal protein S21	rpsU	1.6	0.017	2.04
	A0A2D1CK30	30S ribosomal protein S10	rpsJ	1.45	0.001	1.57
	A0A077VMB7	50S ribosomal protein L21	rplU	1.42	0.032	1.38
	Q2YYQ1	50S ribosomal protein L22	rplV	1.39	0.009	2.57
	A0A0H2HWH9	50S ribosomal protein L14	rplN	1.24	0.036	1.83
	Q6GJC3	30S ribosomal protein S12	rpsL	0.81	0.041	0.45
Glycolysis	A0A0E0VRM3	Acetyl-coenzyme A synthetase	ST398NM01_1785	0.62	0.001	0.23

Oxidative phosphorylation	A0A0E1XGX4	Succinate dehydrogenase iron-sulfur subunit	sdhB	0.82	0.013	0.69
ABC transporters	A0A454GWW4	SAV1866 family putative multidrug efflux ABC transporter (Fragment)	C7P97_12990	1.26	0.012	3.09
	A0A0E1XEH6	Periplasmic binding protein	HMPREF0769_10977	1.25	0.049	1.71
	A0A0E1XHE1	ABC transporter, ATP-binding protein	HMPREF0769_11391	0.59	0	0.18
	A0A2X2K196	Response regulator ArlR	arlR_1	0.66	0.001	0.56
	Q6GFH6	Low molecular weight protein-tyrosine-phosphatase PtpA	ptpA	0.58	0.025	0.31
	A0A1Q8DED0	D-alanyl carrier protein	dltC	0.44	0.047	0.06

Table 1 shows the 26 affected proteins related to 7 biological processes & metabolic pathways. All the TMT results are statistically significant($p<0.05$) and are confirmed by PRM analysis. The ratio of down-regulated proteins in YACTH group compared to the control group is highlighted in green.

Discussion

SAU is a gram-positive bacterium that causes many diseases [18], the current therapeutic strategies against SAU mainly target the cell envelope, protein synthesis, and nucleic acids biosynthesis of the bacteria [19]. With the extensive use of antibiotics, the emergence of drug-resistant strains has become a great challenge for their treatment, which urges people to explore new antibiotics [20]. The herbal formular of YACTH is widely used in Southern China to prevent post-traumatic infection which is commonly caused by SAU [21,22]. In the present study, proteomic analysis was used to analyze the effects of YACTH on the protein expression of SAU. The expressions of 490 proteins were significantly changed in YACTH treated group compared to the control group. These altered proteins were widely associated with a variety of biological processes and multiple metabolic pathways. Interestingly, the down-regulated proteins were mainly involved in the pathways related to RNA polymerase (RNAP), oxidative phosphorylation, and two-component system. The RNAP multisubunit complex is responsible for bacterial transcription [23]. It contains α (two copies), β, β' and ω subunit (Fig 5). The small ω subunit, namely rpoZ, plays key roles in subunit folding, complex assembly, and maintenance of transcriptional integrity [24]. The δ factor, rpoE, plays an essential role in RNA polymerase function in SAU [25]. The ablation of rpoE resulted in decreased transcription, protein synthesis, and pathogenicity [26]. The decreased expression of rpoZ and rpoE in SAU exposed to YACTH suggested that it may inhibit SAU's activities on transcription level by RNAP pathway. In addition, YACTH significantly increased the level of the DEAD-box RNA helicase cshA, which was responsible for RNA degradation and might regulate the expression of virulence factors [27].

Figure 5. RNA polymerase of bacteria and eukaryote.

YACTH significantly down-regulated the expression of several proteins in two-component system and oxidative phosphorylation pathways, including arlRs, ptpA, dltC, and sdhB, etc (Table1), which were potential antibiotics targets associated with bacterial virulence and biofilm formation of SAU [28,29]. The two-component system signal transduction plays a pivotal role in regulating virulence gene expression, cell wall synthesis, biofilm formation, by which bacteria adapt to the external environment [30]. The biofilm formation of SAU is closely related to the intercellular communication and self-defense of the bacteria against antibiotics [31]. For example, arlRs is an antibiotic target for oxacillin, deletion of the arlRs in SAU increased its susceptibilities to oxacillin [32]. The protein tyrosine phosphatase ptpA is a signaling molecule and may interfere with host cell signaling [29,33], which contributes to the infectivity of SAU [34]. Deletion of ptpA not only impaired the survival rates of SAU in macrophages but also reduce the SAU infectivity on mice [29]. DltC, a two-component system protein that affects the cell surface charge of bacteria, is involved in the D-alanine substitution of lipoteichoic acid, and therefore biofilm formation [35]. dltC regulates D-alanine incorporation into cell wall polymers in SAU. A lack of this decoration leads to increased susceptibility to cationic antimicrobial peptide [36,37]. Another downregulated protein associated with biofilm formation is sdhB, a succinate dehydrogenase iron-sulfur subunit involved in the oxidation phosphorylation pathway[38]. The sdhB gene knockout resulted in a 1.2 fold significant decrease of biofilm formation[39], while mutation of sdhB led to defect in the persistence of levofoxacin[38].

On other hand, the up-regulated proteins were primarily involved in the pathways related to mismatch repair, ribosome, aminoacyl-tRNA biosynthesis, and ABC transporter (Table 1). The up-regulated mismatch repair proteins included pcrA,

recJ, and ligA. ATP-dependent DNA helicase pcrA is an essential helicase in Gram-positive bacteria, genetic induction experiment indicated that overproduction of pcrA has deleterious effects on the host cell and plasmid replication [40]. Single-stranded-DNA-specific exonuclease recJ is involved in a variety of DNA repair pathways corresponding to their cleavage polarities, but the relationship between the cleavage polarity and the respective DNA repair pathways has not been clarified [41]. NAD-dependent DNA ligase (ligA) has been thought of as a potential broad-spectrum antibacterial target [42,43,44] and was later proved to be an antibacterial target of SAU and inhibited by pyridochromanone[42]. Our result suggested that YACTH impaired SAU and therefore led to stress response with increased expression of proteins that are involved in mismatch repair. For ribosomes, eighteen ribosomal proteins were upregulated, while only one ribosomal protein was downregulated in YACTH treatment group (table1). Among the 18 upregulated ribosomal proteins, four are related to antibiotics sensitivity, as was reported in previous publications. Mutations of 30S ribosomal proteins S21, rpsU, led to reduced susceptivity of vancomycin of SAU [45]. Increased level of rpIN was also observed in vancomycin-intermediate SAU (VISA) [46]. Mutations on 30S ribosomal proteins S10, rpsJ, was reported to be the cause of reduced tigecycline susceptivity [47]. While mutation of 50S ribosomal protein L22, rplV, is related to several antibiotic resistance, high-level telithromycin selection led to the mutation on rplV [48], mutations of rplV reduced sensitivity of SAU to erythromycin and other macrolides were not only observed in the laboratory but also clinical studies [49,50,51].

As mentioned above, we reported a new antimicrobial herbal formularlation of YACTH and its effects on the expression of bacterial proteins, which might be an alternative choice to protect against post-traumatic infection caused by SAU. The

multiple proteins down-regulated by YACTH were primarily involved in the RNA polymerase, oxidative phosphorylation, and two-component system pathways, which are associated with the biofilm formation and the virulence secretion of SAU. Otherwise, up-regulated proteins by YACTH were mainly presented in the pathways of mismatch repair, ribosome aminoacyl-tRNA biosynthesis, and ABC transporter, etc. Some down- and up-regulated proteins were found to be related to antibiotic sensitivity. These results provided pieces of evidence for the hypothesis that YACTH might repress the growth and infectivity of SAU by either reducing or increasing the expression of target proteins. Some of these proteins were associated with cell envelope, protein synthesis, and nucleic acid biosynthesis, which are pathways that are targets of traditional antibiotics. Unlike common antibiotics that solely mediated on one specific target, YACTH regulated multiple proteins that are involved in several signaling pathways, probably due to its complex components. Such results provided a broad direction of exploring new anti-bacterial targets to fight against SAU. Further studies were needed to clarify the preliminary antibacterial mechanism of each effective component, structure, and potential targets.

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591 Supporting Information

592 **S1 Fig. Subcellular localization of the SAU proteins that were affected by the YACTH**
593 **treatment.**
594 The SAU proteins that were affected by YACTH treatment were assigned into three subcellular
595 locations: cytoplasmic(384), extracellular(87), and membrane(83).

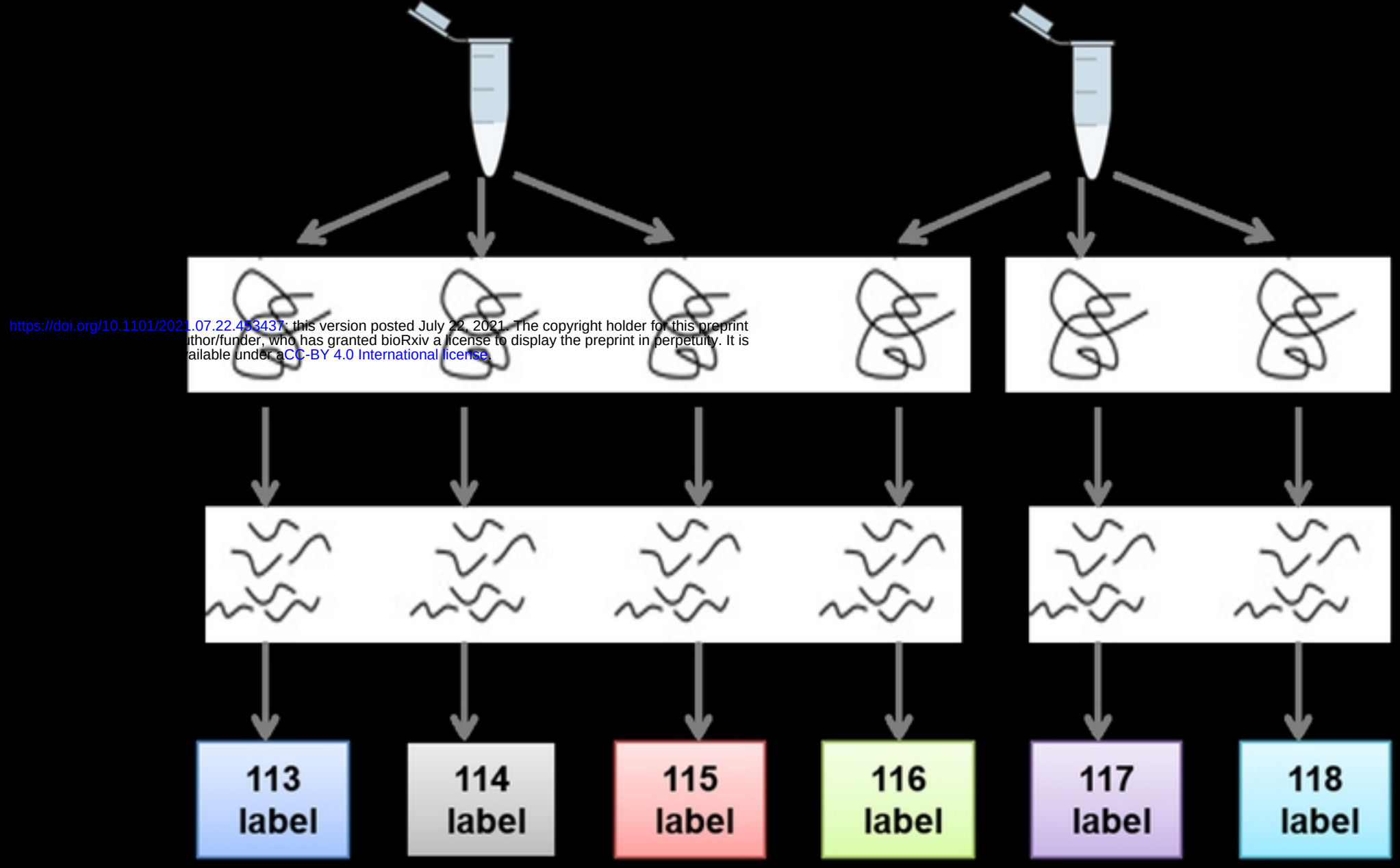


Fig 1

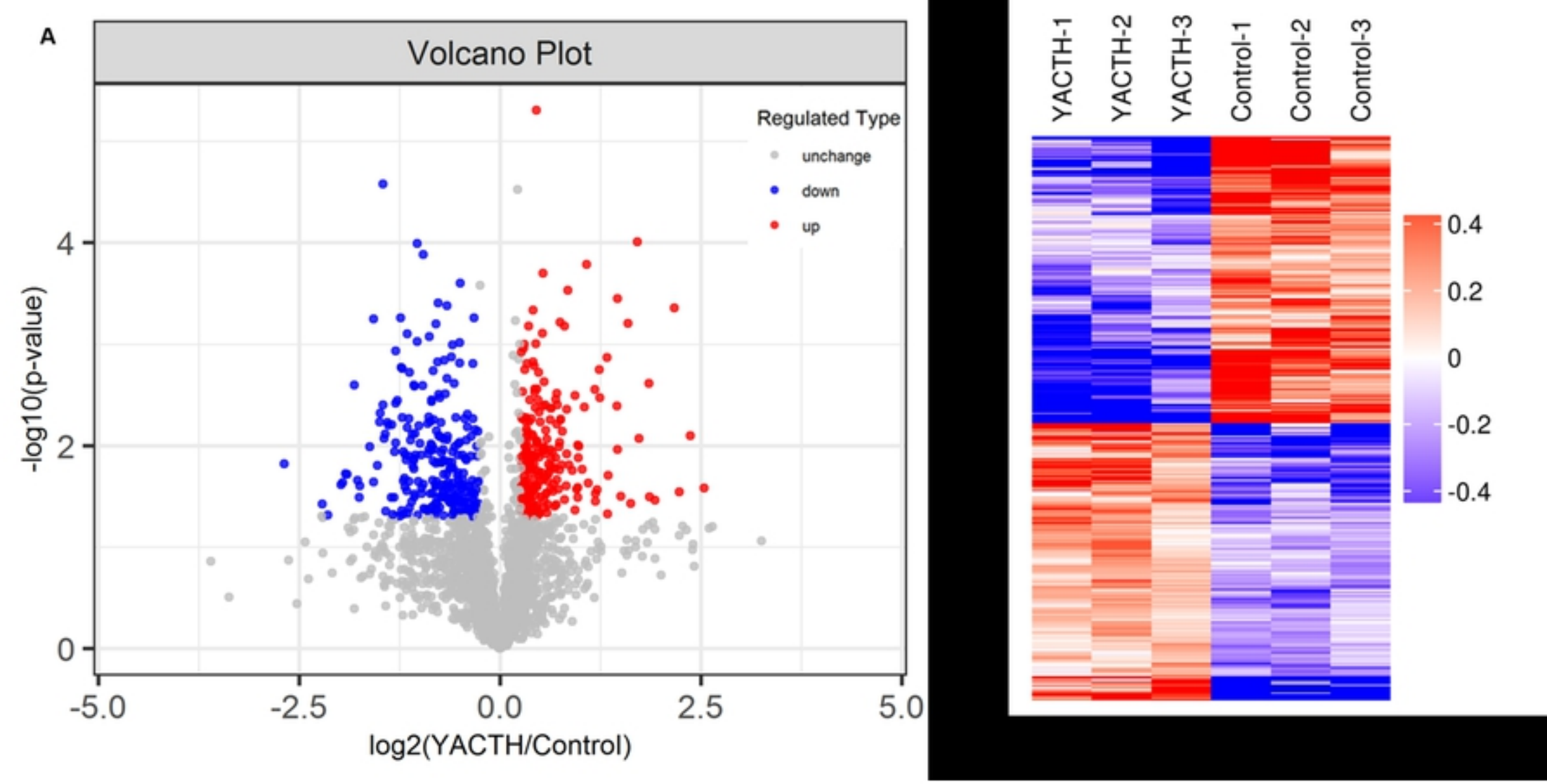

A
Volcano Plot
Regulated Type
unchange
down
up
-log10(p-value)
0
2
4
-5.0
-2.5
0.0
2.5
5.0
log2(YACTH/Control)
YACTH-1
YACTH-2
YACTH-3
Control-1
Control-2
Control-3
0.4
0.2
0
-0.2
-0.4

Fig 2

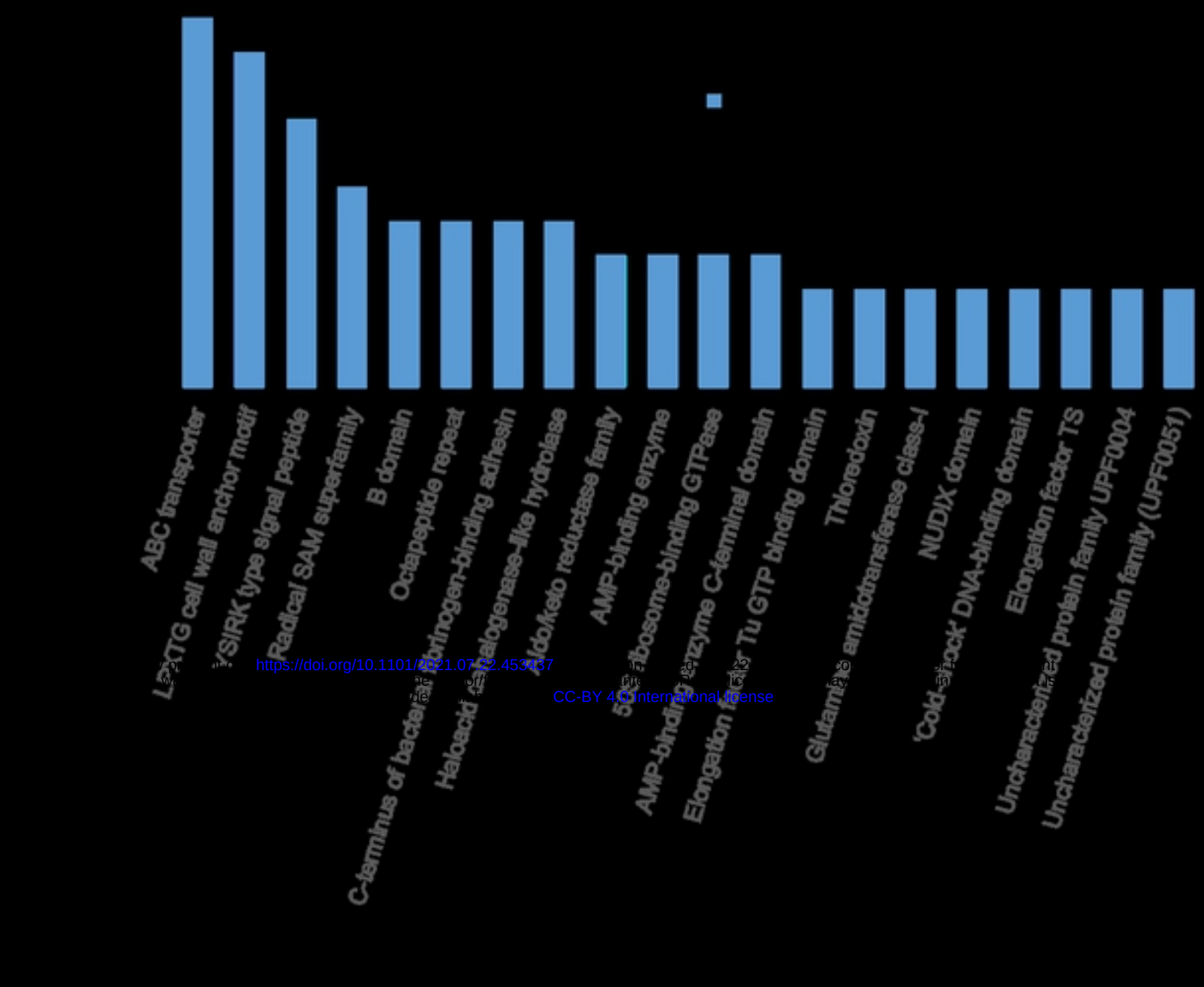


Fig 3

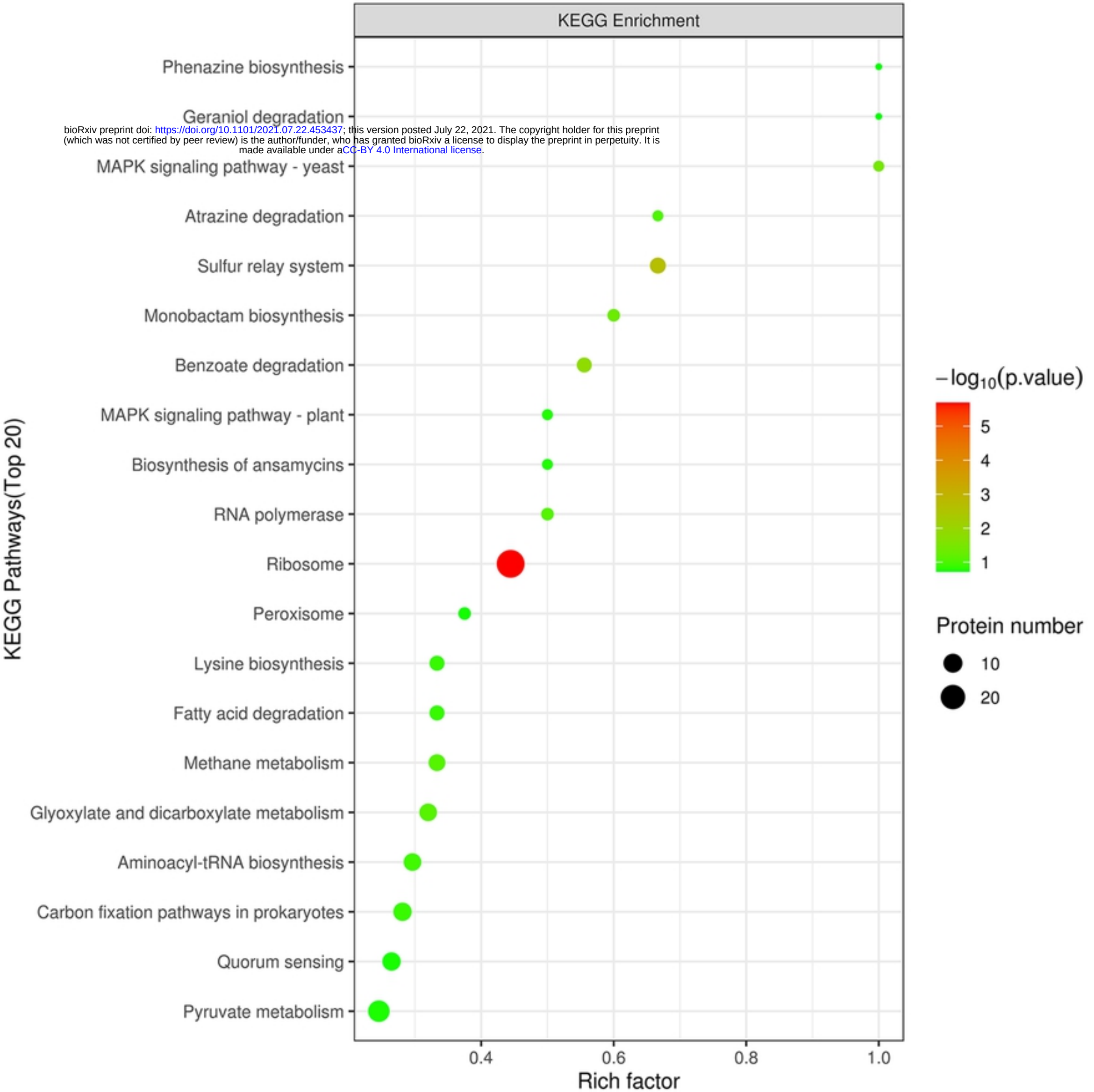
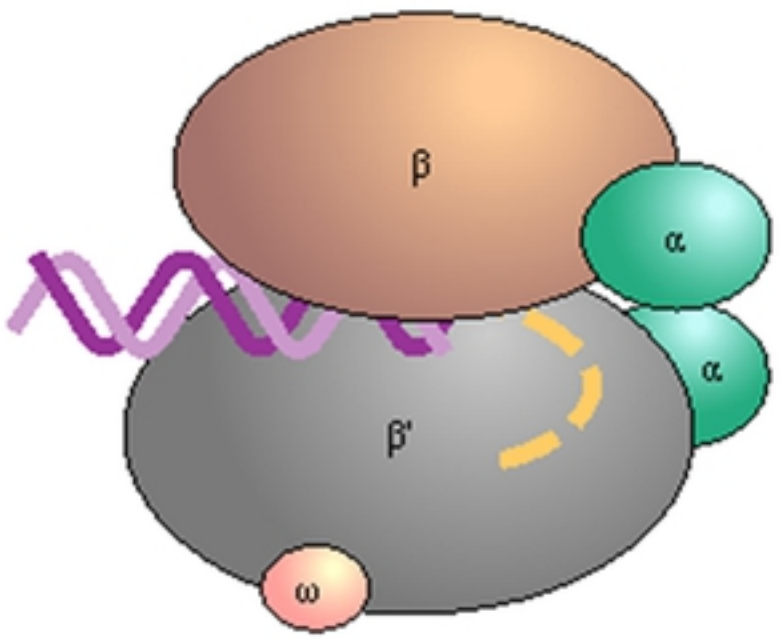
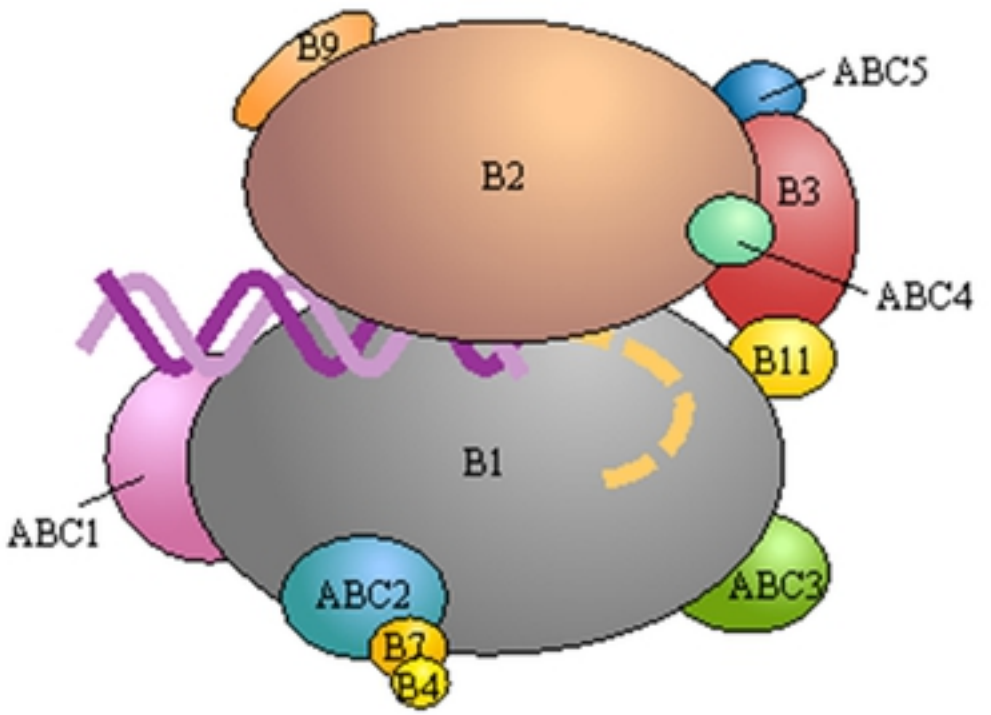


Fig 4

RNA POLYMERASE



RNA polymerase (Thermus aquaticus)



RNA polymerase II (Saccharomyces cerevisiae)

Bacterial

β	α	ω	δ
β'			

Archaeal

B	D	F	H	K	E
A	G		N	L	P

Eukaryotic Pol II

Core subunits

B2	B3
B1	B11

Pol II specific subunits

B4	B7	B9

Pol I, II, and III common subunits

ABC1	ABC2	ABC3
ABC4	ABC5	

Eukaryotic Pol III

Core subunits

C2	AC2
C1	AC1

Pol III specific subunits

C3	C4	C11	
C25	C31	C34	C37

Eukaryotic Pol I

Core subunits

A2	AC2
A1	AC1

Pol I specific subunits

A12	A14	A34
A49	A43	

Fig 5