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Differential protein expression of a streptomycin-resistant *Streptomyces albulus* mutant in high yield production of ε-poly-L-lysine: a proteomics study†

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ε-Poly-L-lysine (ε-PL), produced by *Streptomyces albulus*, is an excellent antimicrobial agent which has been extensively used in the field of food and medicine. In our previous study, we have improved ε-PL production by *S. albulus* M-Z18 through iterative introduction of streptomycin resistance. To decipher the overproduction mechanism of high-yielding mutant *S. albulus* SS-62, we conducted a comparative proteomics analysis between *S. albulus* SS-62 and its parent strain *S. albulus* M-Z18. Approximately 11.5% of the predicted *S. albulus* proteome was detected and 401 known or putative regulatory proteins showed statistically differential expression levels. Expression levels of proteins involved in ε-PL precursor metabolism and energy metabolism, and proteins in the pathways related to transcriptional regulation and translation were up-regulated. It was indicated that mutant SS-62 could not only strengthen the ε-PL precursor metabolism and energy metabolism but also tune the pathways related to transcriptional regulation and translation, suggesting a better intracellular metabolic environment for the synthesis of ε-PL in mutant SS-62. To confirm these bioinformatics analyses, qRT-PCR was employed to investigate the transcriptional levels of *pls*, *frr* and *hrd*D and their transcription levels were found to have increased more than 4-fold. Further, overexpression of *pls* and *frr* resulted in an increase in ε-PL titer and the yield of ε-PL per unit cell. This report not only represents the first comprehensive study on comparative proteomics in *S. albulus*, but it would also guide strain engineering to further improve ε-PL production.

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Introduction

ε-Poly-L-lysine (ε-PL) is a cationic polypeptide made of 25–35 L-lysine residues linked together *via* amide bonds between the ε-amino group and the α-carboxylic acid group. It is mainly produced by bacteria belonging to the Streptomycetaceae family as a secondary metabolite.[1] ε-PL and its hydrochloride form have been used as natural food preservatives in Japan, South Korea, United States, China and other countries because of their excellent antimicrobial properties. In addition, they are applied in numerous other areas as drug carriers, nanoparticles, gene carriers, liposomes, interferon inducers, lipase inhibitors, hydrogels and coating materials.[2] Currently, ε-PL is being industrially produced by aerobic fermentation of *Streptomyces albulus*. However, high production costs remain a major hurdle to widespread use of this natural antimicrobial agent and highly functional material.

Native ε-PL producer *S. albulus* isolated from soil, like other organisms, has an extremely low capability to biosynthesize the target metabolite. Thus, improving the ability of *S. albulus* to synthesize ε-PL is a necessary and effective way to reduce its production cost. Different strategies are being developed to improve microbial production of ε-PL. These include ultraviolet and nitroguanidine,[3] atmospheric and room temperature plasma,[4,5] and genome shuffling.[6,7] Despite these strategies achieving production enhancement of ε-PL, the mechanisms remain unclear and require further investigation. In addition, limitation in the knowledge of genetic information, metabolic pathways and regulatory mechanism restrains significant improvement of ε-PL production through genetic and metabolic engineering of *S. albulus*.[8–10] It is well known that one of the effective ways to reveal the metabolic pathway and regulation mechanism is to analyze the high-yield mutants. However, the mutants obtained by physical or chemical mutagenesis, or genome shuffling are not ideal models since the complexity of their mechanisms, making the changes unknown and variable.

Ribosome engineering, is the introduction of mutations conferring antibiotic resistance to increase metabolite production in bacteria, especially for secondary metabolite production in *Streptomyces*.[11] The mutants resistant to antibiotics frequently possess a point mutation or a deletion mutation

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within a ribosomal component, including ribosomal protein, rRNA, or translation factor.[11] These mutations would change the structure of ribosomes and eventually lead to protein synthesis changes.[12,13] In our previous study, we improved ε-PL production of *S. albulus* M-Z18 by iterative introduction of streptomycin resistance.[14] Despite this, current understanding of this improvement remains limited to biochemistry and gene mutation levels, and it is not clear at the molecular level. However, investigation at the molecular level is crucial for engineering *S. albulus* strains with improved ε-PL production.

In this work, we used comparative proteomics to characterize the differences between parental strain *S. albulus* M-Z18 and high-yield mutant *S. albulus* SS-62 by isobaric tag for relative and absolute quantitation (iTRAQ). Based on identification of potential elements involved in controlling ε-PL production in *S. albulus*, it is possible to further improve the ε-PL production of *S. albulus* by direct genetic manipulation.

Materials and methods

Strains and recombinant plasmids construction

The strains, plasmids, and primers (restriction sites are italicized) used in this study are listed in Table 1. *S. albulus* M-Z18 was isolated from the soil as described by Nishikawa and Ogawa,[15] preserved in our lab, and were subjected to ultraviolet and nitrosoguanidine mutagenesis as described by Hiraki *et al.*[3] *Escherichia coli* DH5α was used as the host for gene cloning and subcloning. *E. coli* strain ET12567 containing pUZ8002 was used as the donor in conjugal transfer of DNA from *E. coli* to *Streptomyces*. All recombinant DNA techniques were performed as described by Sambrook *et al.*[16] pIB139 was used as *E. coli*/*Streptomyces* shuttle vector. Three open reading frames 561-bp *frr*, 3960-bp *pls* and 1002-bp *hrd*D were amplified from *S. albulus* M-Z18 genome DNA by using primers in Table 1, respectively, PCR amplification was started at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s, and elongation at 72 °C for 30 s. The obtained 561-bp *frr* gene encoded a 186 aa long protein, showing the highest similarity to Frr of *S. coelicolor* A3(2) (89%, identical amino acids). The PCR fragments were then digested with *Nde*I and *Eco*RI, and ligated into vector pIB139 that had been digested by the same enzymes. The genes *frr*, *pls* and *hrd*D were inserted into pIB139 to create plasmids pIB139-*frr*, pIB139-*pls* and pIB139-*hrd*D (ESI Files 1 and 2†). The plasmids pIB139-*frr*, pIB139-*pls* and pIB139-*hrd*D were introduced into *E. coli* strain ET12567/pUZ8002 and then transferred into *S. albulus* M-Z18 by intergeneric conjugation to generate recombinant strains.

Culture and fermentation media composition

E. coli strains were cultured in liquid or on solid Luria–Bertani (LB) medium at 37 °C. Apramycin (50 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}), ampicillin (100 μg mL^{-1}), or kanamycin (50 μg mL^{-1}) were added when necessary to maintain the plasmids. *S. albulus* M-Z18 and mutants were maintained on BTN slant medium which contained 10 g L^{-1} glucose, 1 g L^{-1} yeast extract, 2 g L^{-1} peptone, 20 g L^{-1} agar, pH 7.5. The seed medium (M3G) consists of (g L^{-1}): glucose 50, yeast extract 5, $(NH_4)_2SO_4$ 10, K_2HPO_4 0.8, KH_2PO_4 1.36, $MgSO_4 \cdot 7H_2O$ 0.5,

Table 1 Strains, plasmids and primers used in this study

Strains or plasmids or primers	Description	Source or reference
Strains		
E. coli DH5α	General cloning host	This lab
E. coli ET12567 (pUZ8002)	Cm^r, Km^r, donor strain for conjugation	Prof. Wang
S. albulus M-Z18	Wild strain, ε-PL producer	This lab
S. albulus SS-62	Mutant strain, ε-PL producer	This work
M-Z18-*frr*	The M-Z18 strain harboring gene *frr*	This work
M-Z18-*pls*	The M-Z18 strain harboring gene *pls*	This work
M-Z18-*hrd*D	The M-Z18 strain harboring gene *hrd*D	This work
Plasmids		
pIB139	Derivative of integrative plasmid pSET152, harboring a PermE* promoter, Apr^r, $OriT_{RK2}$, ΦC31 int/attP	Prof. Wang
pIB139-*frr*	*frr* gene under the control of promoter PermE* in plasmid pIB139	This work
pIB139 *pls*	*pls* gene under the control of promoter PermE* in plasmid pIB139	This work
pIB139-*hrd*D	*hrd*D gene under the control of promoter PermE* in plasmid pIB139	This work
Primers[a]		
P1	5′-CGCCATATG GTGGTGATCGAAGAGATCCTCC-3′ (NotI)	This work
P2	5′-CGCGAATTC TCAGACCTCTAGGAGCTCGG-3′ (EcoRI)	This work
P3	5′-CGCCATATG ATGTCGTCGCCCCTTCTCG-3′ (NotI)	This work
P4	5′-CGCGAATTC TCACGCGGCCGCACCTCC-3′ (EcoRI)	This work
P5	5′-CCGCATATG ATGGCAACCCGTGCCGTC-3′ (NotI)	This work
P6	5′-CCCGAATTC TCAGGCCGCCGCGTCGAA-3′ (EcoRI)	This work

[a] Primers P1 and P2 were used for amplification of *frr* gene, P3 and P4 were used for amplification of *pls* gene and P5 and P6 were used for amplification of *hrd*D gene.

$ZnSO_4$ 0.04, and $FeSO_4$ 0.03, pH 6.8. The producing medium consists of (g L^{-1}): glycerol 60, $(NH_4)_2SO_4$ 5, yeast extract 8, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 0.5, $ZnSO_4$ 0.04, and $FeSO_4$ 0.03, pH 7.5. All media was sterilized at 115 °C for 15 min.

Fermentation in a 250 mL Erlenmeyer flask

Strains were incubated on BTN slant medium for 8–10 days at 30 °C until spores mature. Three loops of spores were inoculated into a 250 mL Erlenmeyer flask containing 40 mL of seed medium and incubated at 30 °C for 24 h at 200 rpm. Then 8.0% seed medium was transferred into a 250 mL Erlenmeyer flask containing 40 mL of producing medium. Fermentation was carried out at 30 °C for 72 h on a rotary shaker (HYL-C, Qiang Le Laboratory Equipment Co., Taicang, China) at 200 rpm.

Batch fermentation in a 1 l multi-bioreactor system

The batch fermentations were performed in a 1 L glass bioreactor system (T & J Bio-Engineering Co. Ltd., Shanghai, China) consisting of four identical stirred tank reactors with a working volume of 0.6 L. The broth was agitated at 300 rpm with a mechanical stirrer at 30 °C with a 1 vvm aeration rate. The initial pH was adjusted to 6.8 by adding ammonia water (12.5%, v/v), and then the broth was inoculated with 50 mL of 24 h old seed culture. During the fermentation, pH and DO were also monitored online using pH and DO electrodes, respectively (K8S-120 and InPro6800-12-120, Mettler Toledo, Zurich, Switzerland). However, the agitation speed was varied from 300 to 1500 rpm, and aeration rate was manually increased stepwise with steps of 0.5 vvm and a range of 1.0–3.0 vvm.

Morphological characterization of strain by scanning electron microscopy

The morphological characteristics of aerial hyphae and spores of the *S. albulus* M-Z18 and mutant SS-62 were observed after 2 and 7 days of incubation at 30 °C by using scanning electron microscopy (SEM). Cut agar blocks were washed twice in phosphate-buffered saline solution and then immersed in 5% glutaraldehyde. After that, the samples were immobilized with 1% osmium acid (0.1 M PBS, pH 7.2) and then washed with 0.1 M PBS. After an ethanol gradient dehydration, samples were taken at the critical point dried, and pasted on the sample table of the ion sputtering instrument (SCD 005, BAL-TEC, California, America) was coated and scanned under scanning electron microscope (Quanta 200, FEI, Hillsboro, America).

GUS assay

Plasmid PIB139-gusA* harboring the gusA gene was a gift from Dr Ma from China Jiliang University (Hangzhou, Zhejiang). The plasmid PIB139-gusA* was introduced into *E. coli* strain ET12567/pUZ8002 and then transferred into *S. albulus* M-Z18 and mutant strain SS-62 by intergeneric conjugation to generate *S. albulus* M-Z18-GUS and SS-62-GUS, respectively. Mycelia were harvested by centrifugation, washed once with distilled water, and resuspended in lysis buffer (50 mM phosphate buffer [pH 7.0], 5 mM dithiothreitol [DTT], 0.1% Triton X-100, 1 mg mL^{-1} lysozyme). Lysis was performed at 37 °C for 15 min. Lysates were centrifuged at 4000 × *g* for 10 min. Then, 0.5 mL of lysate was mixed with 0.5 mL of dilution buffer [50 mM phosphate buffer (pH 7.0), 5 mM DTT, 0.1% Triton X-100] supplemented with 5 μL 0.2 M *p*-nitrophenyl-β-*d*-glucuronide. The optical density at 415 nm was measured after 20 min of incubation at 37 °C. As a reference, a 1 : 1 mixture of lysate and dilution buffer was used.[17]

Sample preparation, iTRAQ labeling and LC-MS/MS analysis

Ten mL of cultures grown for 30 h were harvested by centrifugation at 10 000 × *g*, 4 °C for 15 min to remove spent media, following which the pellets were washed three times with 50 mm PBS (pH 7.2) for protein extractions. SDT buffer was added to the sample, and transferred to 2 mL tubes with amount quartz sand (another 1/4 inch ceramic bead MP 6540-424 for tissue samples). The lysate was homogenized by MP homogenizer (24 × 2, 6.0 M S^{-1}, 60 s, twice). The homogenate was sonicated and boiled for 15 min. The boiled homogenate was centrifuged at 14 000 × *g* for 40 min and the resulting supernatant was filtered with 0.22 μm filters. The protein content in the filtrate was quantified using the BCA Protein Assay Kit (Bio-Rad, USA). 200 μg of proteins for each sample were incorporated into 30 μL SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris–HCl pH 8.0). The detergent, DTT and other low-molecular-weight components were removed using UA buffer (8 M urea, 150 mM Tris–HCl pH 8.0) by repeated ultrafiltration (Microcon units, 10 kD). Then 100 μL iodoacetamide (100 mM IAA in UA buffer) was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filters were washed with 100 μL UA buffer three times and then 100 μL dissolution buffer (DS buffer) twice. Finally, the protein suspensions were digested with 4 μg trypsin (Promega) in 40 μL DS buffer overnight at 37 °C, and 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 ID 7 mm, volume 3 mL, Sigma), concentrated by vacuum centrifugation and reconstituted in 40 μL of 0.1% (v/v) formic acid. The peptide content was estimated by UV light spectral density at 280 nm using an extinctions coefficient of 1.1 of 0.1% (g l^{-1}) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins. 100 μg peptide mixture of each sample was labeled using iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems).

iTRAQ labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare). The dried peptide mixture was reconstituted and acidified with buffer A (10 mM KH_2PO_4 in 25% of ACN, pH 3.0) and loaded onto a PolySULFOETHYL 4.6 × 100 mm column (5 μm, 200 Å, PolyLC Inc, Maryland, U.S.A.). The peptides were eluted at a flow rate of 1 mL min^{-1} with a gradient of 0–8% buffer B (500 mM KCl, 10 mM KH_2PO_4 in 25% of ACN, pH 3.0) for 22 min, 8–52% buffer B during 22–47 min, 52–100% buffer B during 47–50 min, 100% buffer B during 50–58 min, and buffer B was reset to 0% after 58 min. The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. The collected fractions were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed ID 7 mm, volume 3 mL, Sigma) and

concentrated by vacuum centrifugation. Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto 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) at a flow rate of 300 nL min^{-1} controlled by IntelliFlow technology.

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Thermo Fisher Scientific). The mass spectrometer was operated in positive ion mode. MS data was acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 *m*/*z*) for HCD fragmentation. Automatic gain control (AGC) target was set to 3×10^6, and maximum inject time to 10 ms. Dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70 000 at *m*/*z* 200 and resolution for HCD spectra was set 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%.

Gene expression analysis by quantitative real-time PCR

Total RNAs of *S. albulus* M-Z18 and SS-62 at 30 h (early stationary phase) during fermentation were extracted and purified by RNA extraction kit (MiniBEST, TaKaRa Biotechnology Company, Dalian, China). RNAs were reverse transcribed using a PrimeScript RT Reagent Kit (TaKaRa Biotechnology Company, Dalian, China) according to the manufacturer's instructions. Quantitative RT-PCR was carried out by mixing SYBR Premix Ex Taq (10 μL), forward primer (0.4 μM, 0.8 μL), reverse primer (0.4 μM, 0.8 μL), ROX (0.4 μL), and cDNA (100 ng, 2 μL); a final volume of 20 μL was prepared by adding ddH_2O. The mixture was amplified using a LightCycler®48 II (Applied Biosystems, USA) under the following parameters: 10 min at 94 °C, followed by 40 cycles of 94 °C for 15 s, 60 °C for 1 min. All of these operations were repeated three times and quantitative RT-PCR results were subjected to $2^{-\Delta\Delta C_t}$ method[18] for relative quantification with 16S rDNA as endogenous control gene. Primers used for quantitative RT-PCR are listed in Table 2 and designed by Primer 5.0.

Data analysis

MS/MS spectra were searched using MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4. Protein identification was performed according to the database of Universal Protein (UniProt). The differential proteins of fold change >1.2 or <0.83 and $p < 0.05$ was finalized by the use of biological replicate method. Each protein function was identified by the Gene Ontology (GO) terms and classified by the GO enrichment analysis approach (http://www.geneontology.org/). The online reference system Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for the systematic interpretation of differentially expressed proteins (http://geneontology.org/).

Analytical methods

The samples were obtained from the Erlenmeyer flasks and bioreactor for analysis. The broth was centrifuged (4500 × *g*, 10 min), and the precipitate was collected, washed twice with distilled water, and then dried at 105 °C to a constant weight to determine dry cell weight (DCW) of the culture. The concentration of glucose was determined using a biosensor (SBA-40C, Biology Institute of Shandong Academy of Sciences, Shandong, China). The supernatant was used to determine the ε-PL concentration using the methyl orange precipitation method according to Itzhaki.[19] In brief, an equal volume of sample with 0.06–0.12 g L^{-1} ε-PL diluted with 0.07 mM phosphate buffer (pH 6.90) and 0.7 mM methyl orange solution were mixed together, which were reacted at 30 °C with shaking for 30 min. The ε-PL concentration can be estimated from the absorbance at 465 nm of the methyl orange remaining in the supernatant solution through standard curve calculation.

Statistical analysis

All described experiments were performed in independent biological triplicates, unless stated otherwise. Presented data in the graphs are the averages of the replicates ± the standard deviation. Differences were considered statistically significant if the *p* value from *t*-test (MS Excel 2010) for two samples with equal variance was $p < 0.05$.

Table 2 Sequences of primer pairs designed for qRT-PCR

Gene	Gene product	DNA sequence[a] (5′–3′)
pls	ε-PL synthase	F: TCTTCACGCTCCTGACCACT R: CCGAGGCTGGCGTAGAG
*hrd*D	RNA polymerase sigma factor	F: GCCAAGGAGGTGGAGCTGTCCC R: CGGGCGACGGCGACGACC
frr	Ribosome recycling factor	F: CAACATCATCCGTGTGAC R: CTTGTTCTTGGCGACCTT
relA	ppGpp	F: GCATGAACACCATCAAGT R: CGATCTCGTCGTACATCT
16S rDNA	Control gene	F: CAGGCTAGAGTTCGGTAG R: CGGTGTTCCTCCTGATAT

[a] All primers were designed according to the sequence of the *S. albulus* M-Z18.

Results and discussion

Fermentation characteristics and growth behavior of M-Z18 and SS-62

Bioassays including ε-PL concentration quantification, DCW and ε-PL/DCW analysis of culture supernatants from the two strains were performed at 96 h. As shown in Fig. 1, the ε-PL concentration detected in the culture supernatants from the SS-62 strain attained 3.04 g L^{-1} in shake-flask fermentation, which was a 1.79-fold increase compared to the production in *S. albulus* M-Z18 culture supernatants. Meanwhile, the DCW and ε-PL/DCW of SS-62 were enhanced 1.25-fold and 1.55-fold, respectively.

It is of great value to recognize changes in the cultivation characteristics of high-yielding strain for industrial application.[7,20] ESI File 3† shows the macroscopic and microscopic photographs of M-Z18 and SS-62 in solid and liquid culture. M-Z18 colonies were smooth and pure white, while SS-62 colonies had a volcanic rock shape with a central depression and were surrounded by folds. Further, SEM analysis showed the hyphae of SS-62 appeared full and abundant as the parent strain M-Z18, but no vesicles and the existence of mycelial helixes were observed in SS-62 (ESI Files 3A-1 and B-4†). When the colonies matured, M-Z18 colonies had an abundance of spores, but SS-62 colonies were flat with some white dots spread in the spores. In addition, M-Z18 spores were brown, while SS-62 spores were greyish green. From the SEM analysis, it can be found that SS-62's spores were bigger and had more spines than the parent strain M-Z18 (ESI Files 3A-2, and B-5†). In contrast, there was not much difference between mycelium morphology of M-Z18 and SS-62 in liquid culture (ESI Files 3A-3, and B-6†).

Protein synthesis activity *in vivo*

Luzhetskyy's group has developed a versatile and sensitive reporter system for actinomycetes which is based on gusA encoding β-glucuronidase, and has shown the utility of the GUS reporter system for gene expression study at the translation level in actinomycetes.[17] GFP was used for the evaluation of protein synthesis in recombinant strains in an earlier study,[21] but its reliability when investigating protein synthesis in actinomycetes is unclear. Therefore, to determine the relationship between protein synthesis and ε-PL production in the parental strain and mutants, a reporter gene assay based on glucuronidase gene (GUS) was performed. The glucuronidase activity was measured at 24, 48 and 72 h after inoculation. As shown in Fig. 2, mutant SS-62 exhibited higher glucuronidase activity compared to that of parental strain M-Z18 at 48 and 72 h. It indicated that the high-level ε-PL production may be caused by accelerated protein synthesis.

Sampling conditions and summary of proteomics analysis

To understand the potential elements involved in controlling ε-PL production in *S. albulus*, comparative proteomics based on iTRAQ analysis was performed. It is well known that the synthesis of ε-PL is closely related to pH.[22] To exclude the impact of pH, 30 h shake-flask fermentation broth was chosen as sample to obtain a proteomic profile at the beginning of ε-PL synthesis. A list of amino acid sequences of all proteins predicted to be encoded by *Streptomyces albulus* PD-1 chromosome and plasmids was compiled based on previously reported genome sequences (ESI File 4†).[23]

iTRAQ experiments identified 3486 proteins from *S. albulus* according to UniProt database. Overall 401 proteins (11.50%) in SS-62 were significantly differentially expressed compared to expression in M-Z18. Of these proteins, 188 proteins (46.88%) were up-regulated and 213 proteins (53.12%) were down-regulated (Fig. 3A, ESI File 5†). GO analysis revealed the first

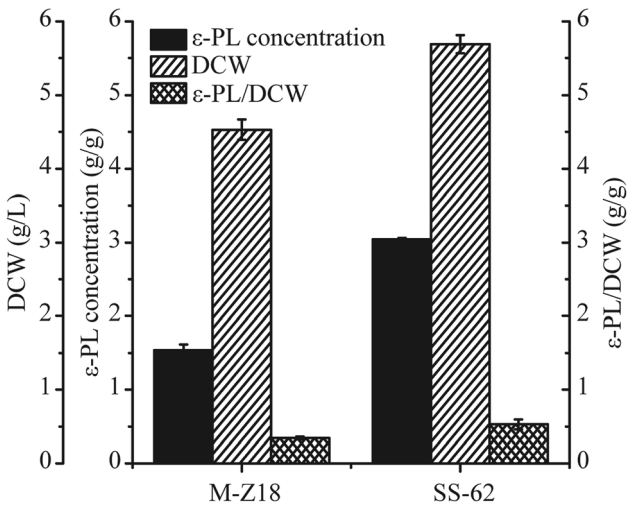

Fig. 1 Fermentation parameters of parent strain *S. albulus* M-Z18 and mutant strain SS-62 in Erlenmeyer flask. Data represents the means of three independent experiments, and error bars represent the standard deviation.

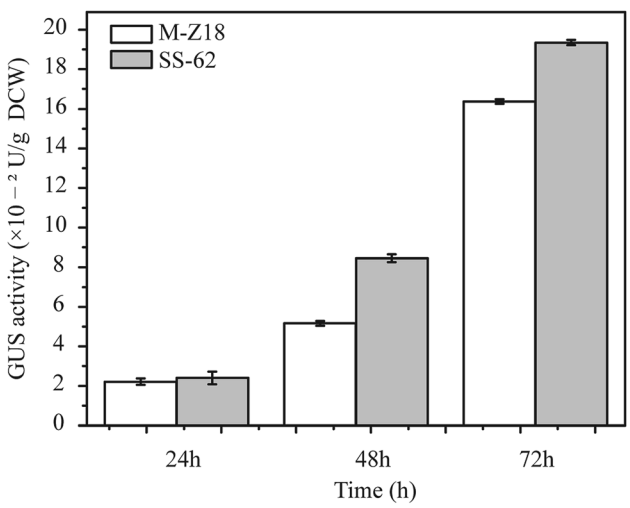

Fig. 2 Detection of β-glucuronidase activity. Glucuronidase activity was measured in cell lysates of parental strain *S. albulus* M-Z18 and mutant SS-62 from fermentation broth at 24, 48 and 72 h, respectively. The data shown are the mean values of three replicates ± standard deviation (SD), which is indicated by an error bar.

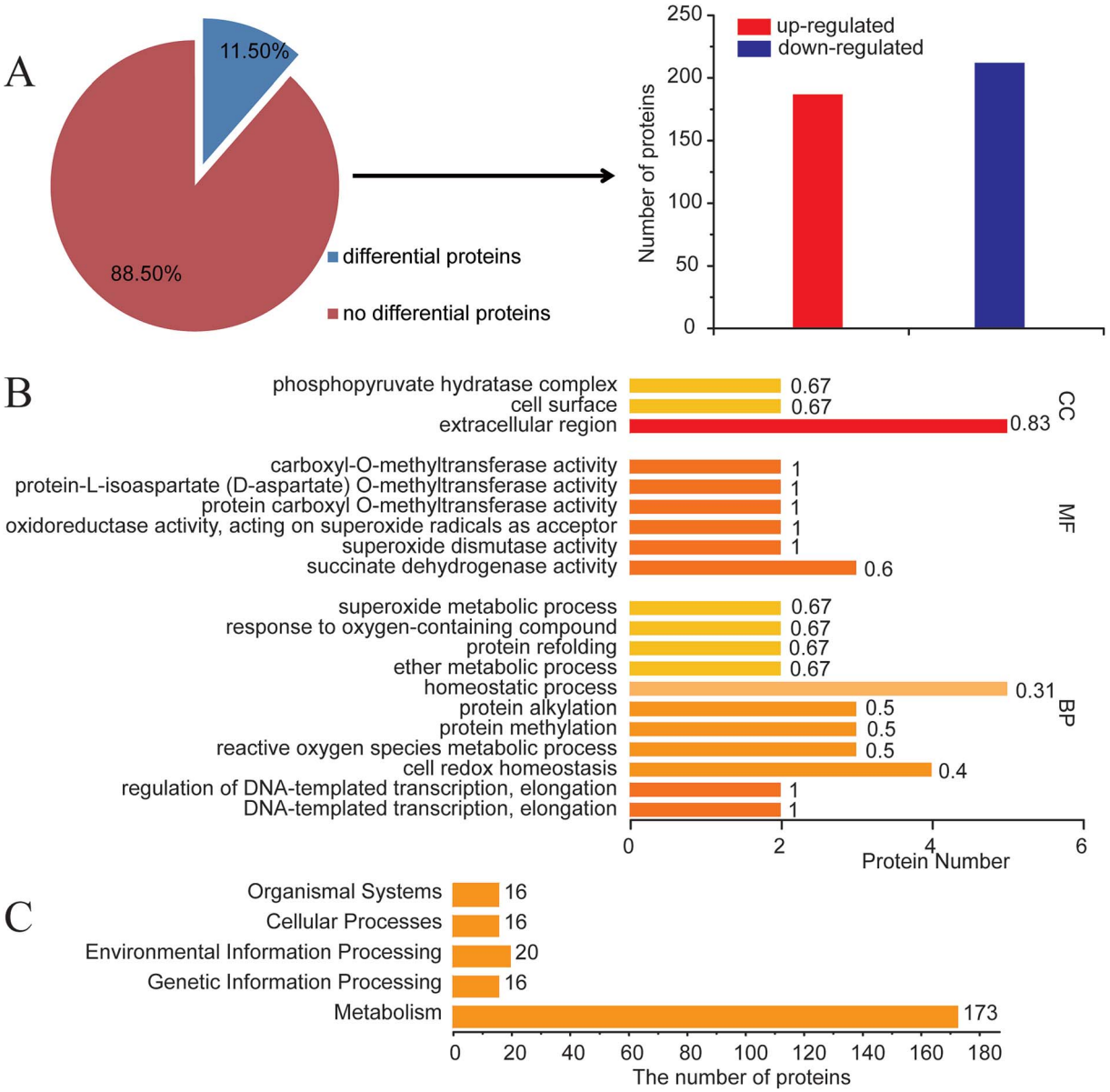

Fig. 3 Proteins identified by searching UniProt and GO enrichment analysis. Numbers of the detected proteins (3486) of *S. albulus*, of which 11.50% are differentially expressed proteins (401) compared with the patent strain (A). The first 20 significant biological functional pathways of GO analysis related to the percentage of the differential proteins (B). Differential proteins related to the different biological functions by KEGG analysis (C).

20 significant biological functional pathways of the differential proteins (Fig. 3B).

KEGG analysis results showed some different biological functional properties (Fig. 3C) on the basis of differential proteins, including organismal systems, cellular processes, environmental information processing, genetic information processing and metabolism. Of particular interest in this study was the identification of metabolic and regulatory proteins that may be directly or indirectly regulation ε-PL biosynthesis in SS-62. This includes carbohydrate metabolism, amino acid metabolism, energy metabolism and other transcriptional regulators.

Changes of carbohydrate metabolism, amino acid metabolism and energy metabolism in SS-62

Carbohydrate metabolism, amino acid metabolism and energy metabolism are essential for ε-PL synthesis. ε-PL synthesis was strengthened from carbon metabolism and energy metabolism with 0.5% *n*-dodecane in broth.[24] Results indicated that most of the differential proteins were mainly related to metabolism and genetic information processing (Fig. 4A). However, the proteins associated with carbohydrate metabolism, amino acid metabolism and energy metabolism accounted for most of the differential proteins. Carbohydrate metabolism is a crucial important pathway for cellular activities.[25] The pathway mainly includes PPP, EMP,

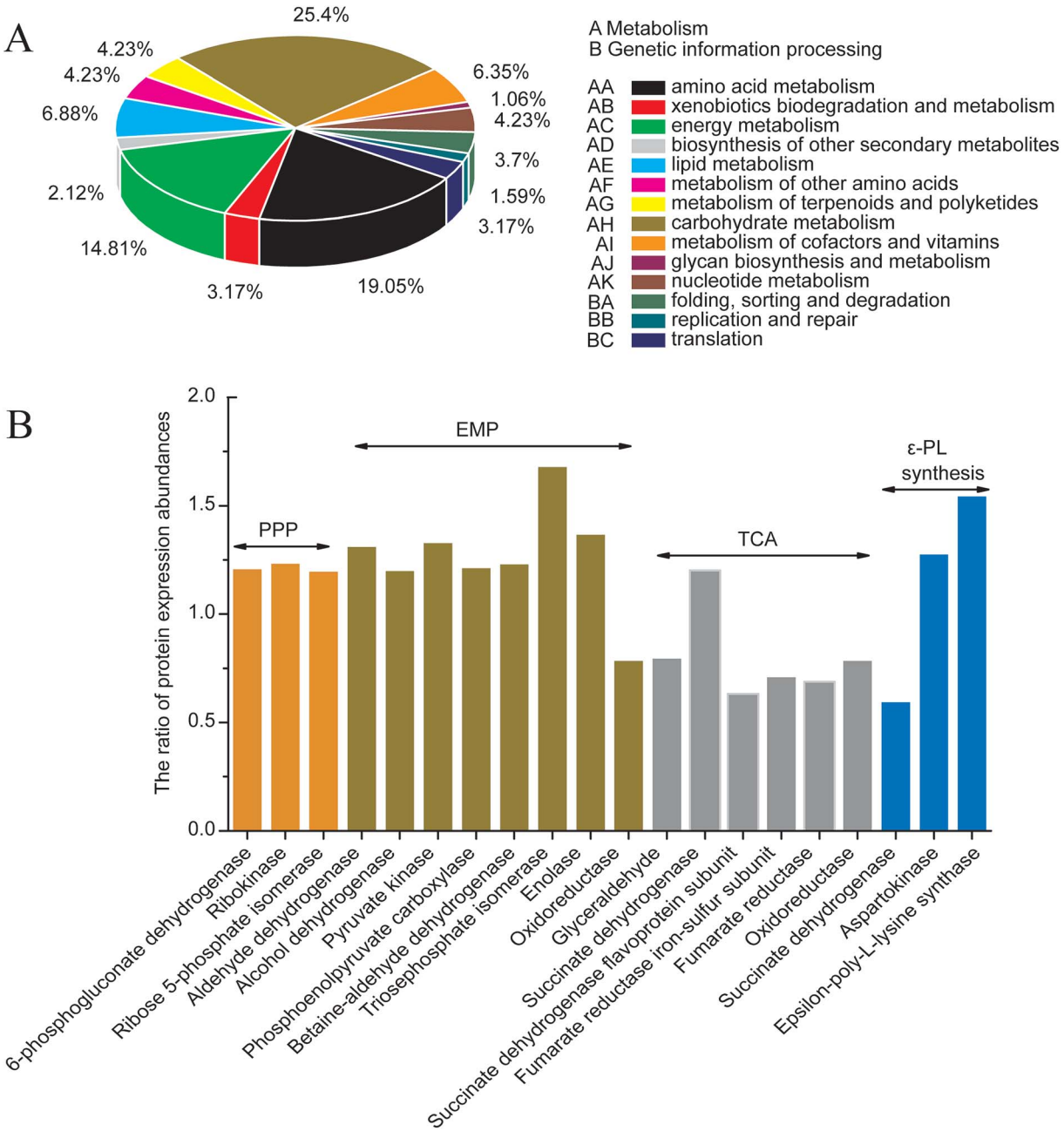

Fig. 4 Differential proteins related to the metabolism and genetic information processing by KEGG analysis.

TCA, DAP and anaplerotic pathway. Differentially expressed proteins from iTRAQ results were displayed in Fig. 4B and ESI File 6.† G6PDH (A0A059VZG9) is the rate-limiting enzyme in the pentose phosphate pathway, which is responsible for providing NADPH and pentoses used for cell growth and metabolites biosynthesis.[26] The expression of G6PDH in SS-62 was increased 1.21-fold. PEPC (A0A059W8H1) and PYC (X0MXA7) catalyze conversion of phosphoenolpyruvate and pyruvate into oxaloacetic acid in the anaplerotic pathway, respectively, as a supplementary to tricarboxylic acid cycle. Results showed that the expression levels of both proteins were enhanced 1.22- and 1.3-fold, respectively. It is reported that L-lysine, the precursor to ε-PL, is synthesized through diaminopimelate pathway in bacteria.[2] This pathway is initiated from the phosphorylation of aspartate by the catalytic activity of Ask. As shown in Fig. 4B, the expression of ASK (Q0KJ33) was up-regulated by 1.28-fold. *pls* is a nonribosomal peptide synthetase that catalyzes the polymerization of L-lysine to produce ε-PL with consumption of ATP.[27,28] Results showed that the expression of Pls (B5BR95) in SS-62 was significantly increased, about 1.55-fold. These data show iterative introduction of streptomycin resistance significantly increased the protein expression related to cell growth and ε-PL synthesis in mutant SS-62.

Amino acid metabolism and energy metabolism were compared between SS-62 and M-Z18 (ESI File 6†). The

metabolism of histidine (X0N9B5, X0MQF6, X0MXT6, X0N359) and arginine (X0NZK5, X0N514) were strengthened in SS-62. It is possible that after continuous stimulation with streptomycin, basic amino acids were more readily synthesized and the cells had a stronger ε-PL synthesis capacity. However, lysine degradation ability was enhanced, and the reduced lysine might be used for cell growth and ε-PL synthesis. Phenylalanine (A0A059W7X8, X0MY85), tyrosine and tryptophan biosynthesis (X0MZG0, X0N525), glycine, serine and threonine metabolism (A0A059WCN5, X0MW61, A0A059W2E1), alanine, glutamic acid (X0N4C0, A0A059W6Z3) and other metabolic byproducts had been weakened. This result indicated that the synthesis of byproducts decreased in the process of ε-PL synthesis in SS-62. Moreover, some increased expression of proteins associated with oxidative phosphorylation (X0NA06, A0A1A9QKT3, X0MJ64), carbon fixation pathways in prokaryotes (A0A059VUK9, X0NA06), and methane metabolism (A0A059W0D7, X0MSW1, A0A059WCI8, A0A059VUK9, A0A1A9QG52) also suggest that SS-62 synthesizes more energy and may promote the synthesis of ε-PL.

Translation and related proteins

Several proteins that are known or may be involved in regulating ε-PL production were detected in the dataset (Table 3). Expression of one glutamyl-tRNA^{Gln} amidotransferase family protein (X0N7H0, A0A059W8F5/GatA) was increased 1.22-fold in SS-62 compared with the parental strain. X0N571 was increased 1.36-fold. The decrease of 30S ribosomal protein S1 (A0A1A9QG37) expression in SS-62 mutant was not expected as it has a role in the ribosome dynamic properties to initiate translation of a large set of mRNAs with diverse structural features.[29] However, 30S ribosomal protein S6 (A0A059W5Y0) in mutant SS-62 was up-regulated 1.89-fold. Interestingly, three elongation factors (X0P1T9, A0A1A9QQA8, X0P2R5) that promote polypeptide chain in mRNA translation were also up-regulated significantly in mutant SS-62, which might be due to the increase in expression of proteins related to ε-PL synthesis. Protein biosynthesis, which occurs on the ribosome, consists of four steps: initiation, elongation, termination, and ribosome recycling. The subsequent translation cycle requires breakdown of the ribosome–mRNA–tRNA posttermination complex and recycling of the ribosomal subunits. The ribosome recycling factor (RRF), together with the elongation factor-G (EF-G), is involved in catalyzing the disassembly of the post-termination complex and recycling the ribosome.[30] Furthermore, RRF, a product of *frr* gene responsible for the dissociation of ribosomes from messenger RNA after the termination of translation, was up-regulated in SS-62. *frr* gene encoding RRF is widely distributed in prokaryotes and eukaryotic organelles, and is present in all prokaryotes examined to date. RRF has been shown to strongly stimulate *in vitro* protein synthetic activity in *Escherichia coli*, suggesting that the efficiency of protein synthesis is increased by recycling components of the post-termination complex.[31] An increased expression of RRF in *Streptomyces coelicolor* was recently shown to cause enhanced protein synthesis during the late growth phase and subsequent overproduction of the antibiotic.[32]

Transcriptional regulators

The expression of secondary metabolic gene clusters is controlled by many different families of regulatory proteins, some of which are found only in actinomycetes, and is elicited by both extracellular and intracellular signaling molecules.[33] A total of 33 transcriptional regulators were detected in iTRAQ dataset (Table 4), which were categorized

Table 3 Comparative expression levels of translation and related proteins during iTRAQ analysis of protein extracts from the wt, and SS-62 strains of *S. albulus*

Accession	Putative function	Protein	Gene	Unique peptides	Peptides	Fold change[a]	*p* Value
Translation							
X0N7H0	Translation	Glutamyl-tRNA (Gln) amidotransferase subunit A	gatA	16	17	1.22	0.0212
X0N571	Regulation of translational fidelity	Isoleucine-tRNA ligase	ileS	25	25	1.36	0.0004
A0A059W8F5	Regulation of translational fidelity	Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit C	gatC	2	2	1.53	0.0225
A0A059W5Y0	Translation	30S ribosomal protein S6	rpsF	5	5	1.89	0.0005
A0A1A9QQ8	—[b]	Peptidase	A4V12_17360	1	1	1.20	0.0104
A0A1A9QG37	Translation	30S ribosomal protein S1	rpsA	1	26	0.71	0.0269
Others							
X0P1T9	Translational elongation	Elongation factor Tu	tuf	14	24	1.35	0.0293
A0A1A9QQA8	Translational elongation	Elongation factor Ts	tsf	19	20	1.36	0.0019
X0P2R5	Translational elongation	Transcription elongation factor GreA	greA	6	6	1.27	0.0367
X0MS37	Translational termination	Ribosome-recycling factor	frr	15	15	1.21	0.0415

[a] Fold change between two strains (mutant SS-62/parent M-Z18), fold change > 1.2 represented upregulation, fold change < 0.83 represented downregulation. [b] Function unknown.

Table 4 Differentially expressed transcriptional regulators in mutant *S. albulus* SS-62 compared with the parent strain *S. albulus* M-Z18

Accession	Putative function	Protein	Gene	Unique peptides	Peptides	Fold change[a]	*p* Value
X0MIN4	—[b]	Crp/Fnr family transcriptional regulator	P354_08015	19	20	1.28	0.0010
A0A059WCR4	Regulation of transcription, DNA-templated	MarR family transcriptional regulator	DC74_5136	9	9	1.21	0.0124
X0MS01	—	Transcriptional regulator	P354_28905	3	3	1.40	0.0050
X0MWP7	Regulation of transcription, DNA-templated	Transcriptional regulator	P354_18815	6	6	1.74	0.0001
X0MRL4	Sequence-specific DNA binding	DNA-binding protein	P354_30280	1	1	1.30	0.0161
X0N564	Integral component of membrane	Transcriptional regulator	P354_27980	1	1	1.25	0.0116
X0N2N8	Oxidation–reduction process	DNA-binding protein	P354_25910	2	2	1.23	0.0024
A0A059WA88	Regulation of transcription, DNA-templated	AsnC family transcriptional regulator	DC74_4159	1	1	1.23	0.0194
A0A059W106	—	MarR family transcriptional regulator	DC74_1054	1	1	1.22	0.0090
A0A059VZR2	Biosynthetic process	Putative GntR family transcriptional regulator	DC74_566	5	5	1.21	0.0145
A0A059WBX8	—	Transcriptional regulator	DC74_4870	5	5	1.22	0.0276
X0N4Z4	—	Transcriptional regulator	P354_28185	9	9	0.78	0.0130
X0NBV0	Regulation of transcription, DNA-templated	TetR family transcriptional regulator	P354_12810	3	3	0.78	0.0212
A0A059WBQ9	Regulation of transcription, DNA-templated	DeoR family transcriptional regulator	DC74_6728	4	4	0.79	0.0335
X0N4A8	Regulation of transcription, DNA-templated	LysR family transcriptional regulator	P354_31270	4	4	0.76	0.0049
X0N5W5	Regulation of transcription, DNA-templated	TetR family transcriptional regulator	P354_27365	2	2	0.81	0.0343
X0N9V3	Regulation of transcription, DNA-templated	LuxR family transcriptional regulator	P354_18990	1	1	0.79	0.0227
A0A059WD02	Regulation of transcription, DNA-templated	TetR family transcriptional regulator	DC74_6856	2	2	0.77	0.018
A0A059WE02	Regulation of transcription, DNA-templated	TetR family transcriptional regulator	DC74_7592	1	1	0.63	0.0142
X0MHH6	DNA binding	DNA-binding protein	P354_13010	3	3	0.82	0.0474
X0MQA0	Regulation of transcription, DNA-templated	GntR family transcriptional regulator	P354_33540	1	1	0.75	0.0028
X0NHJ	Regulation of transcription, DNA-templated	LuxR family transcriptional regulator	P354_00025	2	2	0.79	0.0391
A0A059WFL3	Regulation of transcription, DNA-templated	LysR family transcriptional regulator	DC74_7800	1	1	0.27	0.0079
X0MNE4	Sequence-specific DNA binding	Cro/Cl family transcriptional regulator	P354_37150	2	2	0.76	0.0045
A0A059WH51	Regulation of transcription, DNA-templated	TetR family transcriptional regulator	DC74_6720	1	1	0.78	0.0067
X0N092	Regulation of transcription, DNA-templated	TetR family transcriptional regulator	P354_05815	1	1	0.71	0.0266
A0A1A9QJ90	—	IclR family transcriptional regulator	A4V12_27770	1	1	0.78	0.0098
A0A059VZH0	Regulation of transcription, DNA-templated	MarR family transcriptional regulator	DC74_486	1	1	0.78	0.0067
A0A059W3M0	Regulation of transcription, DNA-templated	Cold shock protein	DC74_3899	1	3	1.68	0.0361
X0NAN3	Regulation of transcription, DNA-templated	Cold shock protein	P354_16170	4	5	1.40	0.0075
X0P2W9	Regulation of transcription, DNA-templated	Cold shock protein	P354_13760	2	3	1.78	0.0091
X0MYX2	Regulation of transcription, DNA-templated	Cold shock protein	P354_42355	2	4	1.79	0.0296
A0A059VY86	Regulation of transcription, DNA-templated	Cold shock protein	DC74_24	2	3	1.28	0.0009

[a] Fold change between two strains (mutant SS-62/parent M-Z18), fold change > 1.5 represented upregulation, fold change < 0.83 represented downregulation. [b] Function unknown.

into 12 significant families and one other cluster. The MarR-family transcriptional regulators (A0A059WCR4, A0A059W106) were up-regulated in mutant SS-62 compared to in the parent strain. In addition, Crp/Fnr family protein (X0MIN4), which is a pleiotropic transcriptional regulator that controls a broad range of cellular functions,[34] was up-regulated in SS-62. Further analysis indicated that the LysR-family transcriptional regulators (X0N4A8, A0A059WFL3) and one single IclR transcriptional regulator detected in dataset were down-regulated in SS-62. And disruption or deletion of orf10 (LysR-type transcriptional regulators) in *Streptomyces lividans* causes actinorhodin overproduction.[35]

Six additional regulators were differentially expressed in the comparison, which included proteins belonging to the AsnC-family (A0A059WA88), Cro/Cl-family (X0MNE4), LuxR-family (X0N9V3, X0NHJ), NmrA-family (497683061) and DeoR-family (490052578). AsnC (A0A059WA88) was up-regulated 1.23-fold than in M-Z18. It is reported that deletion of SCO3361, an Lrp/AsnC family protein from *Streptomyces coelicolor*, led to dramatic reduction in actinorhodin production.[36] A single GntR-family transcriptional regulator (490058535), which has various functions including gluconate repression, and multiple TetR-family transcriptional regulators were all found to be under-expressed in SS-62. TetR-family members are involved in the transcriptional regulation of multidrug efflux, antibiotic biosynthesis pathways, osmotic stress response, catabolic pathways and differentiation processes. Importantly, it has been proven that the TetR family contains transcriptional repressors by Ramos *et al.*[37] The down-regulation of TetR transcriptional regulators may be beneficial to ε-PL synthesis. In addition, five cold shock proteins (A0A059W3M0, X0NAN3, X0P2W9, X0MYX2, A0A059VY86) were significantly over-expressed in the mutant SS-62. Cold shock proteins are small nucleic acid-binding proteins ranging from 65 to 75 amino acids in length, which are not only produced during cold stress but have a much wider role in the stress response of bacteria.[38] It has a function as RNA chaperones by destabilizing secondary structures in target RNA at low temperature so that the single-stranded state of target RNA is maintained, this enables efficient transcription and translation.[39]

qRT-PCR analysis of related genes and overexpression of *frr* and *pls*

We performed gene expression analysis of some genes, *viz.*, B5BR95 (*pls*), X0MS37 (*frr*), A0A059VZL5 (*rel*A), and HrdD (*hrd*D), based on the criteria of fold change observed in the proteomic analysis and involvement in important physiological pathways. As shown in Fig. 5, qRT-PCR results showed that the transcription level of *pls*, *frr*, and *hrd*D in SS-62 were increased at different levels. Especially, the transcription level of *frr* was enhanced by 6.3-fold. Moreover, the transcription levels of *pls* and *hrd*D in the SS-62 mutant were all increased by 4.6-fold, which may be responsible for enhancing ε-PL production. It is reported

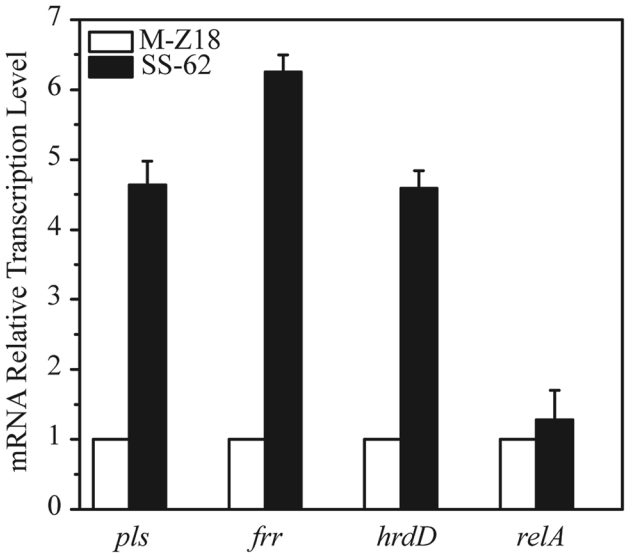

Fig. 5 mRNA expression quantitation of related genes in *S. albulus* M-Z18 and SS-62 upon iTRAQ analysis.

that HrdD can specifically bind to the *pls*-gene promoter, regulate the *pls* expression, and initiate ε-PL synthesis.[40,41] However, there was no difference in the expression of *relA* at transcriptional and protein levels. This was surprising as ppGpp (*relA* gene encoding ppGpp synthetase) is necessary for antibiotic production under conditions of nitrogen limitation in *Streptomyces coelicolor* A3(2)[42] and for cephamycin C production in *Streptomyces clavuligerus*.[43,44] Therefore, whether ppGpp was directly involved in promoting transcription of ε-PL biosynthetic genes was unclear.

Proteomics and qRT-PCR results showed that the expression of Pls, RRF and HrdD in SS-62 were all increased. Herein, we investigated the positive role of Pls, RRF and HrdD in ε-PL production through overexpression of *pls*, *frr* and *hrd*D genes in *S. albulus* M-Z18, respectively. As shown in Fig. 6, the results indicated that there is no difference in the consumption rate of glucose between M-Z18 and M-Z18-*pls* (Fig. 6A). But the ε-PL production and the synthesis of ε-PL per unit cell of M-Z18-*pls* increased by 7.2% and 20.3% compared to M-Z18, respectively (Fig. 6C and D). However, the consumption rate of glucose and the DCW by *S. albulus* M-Z18-*frr* was slower than M-Z18 (Fig. 6A and B). Although the ε-PL production of *S. albulus* M-Z18-*frr* increased slightly, the ε-PL synthesis of M-Z18-*frr* was always higher than that of M-Z18 before 30 h, and the synthesis of ε-PL per unit cell increased significantly (Fig. 6C and D). Hosaka *et al.*[32] and Ma *et al.*[45] showed that an increased expression of RRF in *S. coelicolor* and *S. diastatochromogenes* 1628 can enhance the production of antibiotics, which were consistent to our results. However, the detailed mechanism of up-regulated *frr* to increase antibiotic production remains to be clarified. Unfortunately, the overexpression of *hrd*D in *S. albulus* M-Z18 was not made difference in terms of DCW, ε-PL production and the synthesis of ε-PL per unit cell than the *S. albulus* M-Z18 (ESI File 2†).

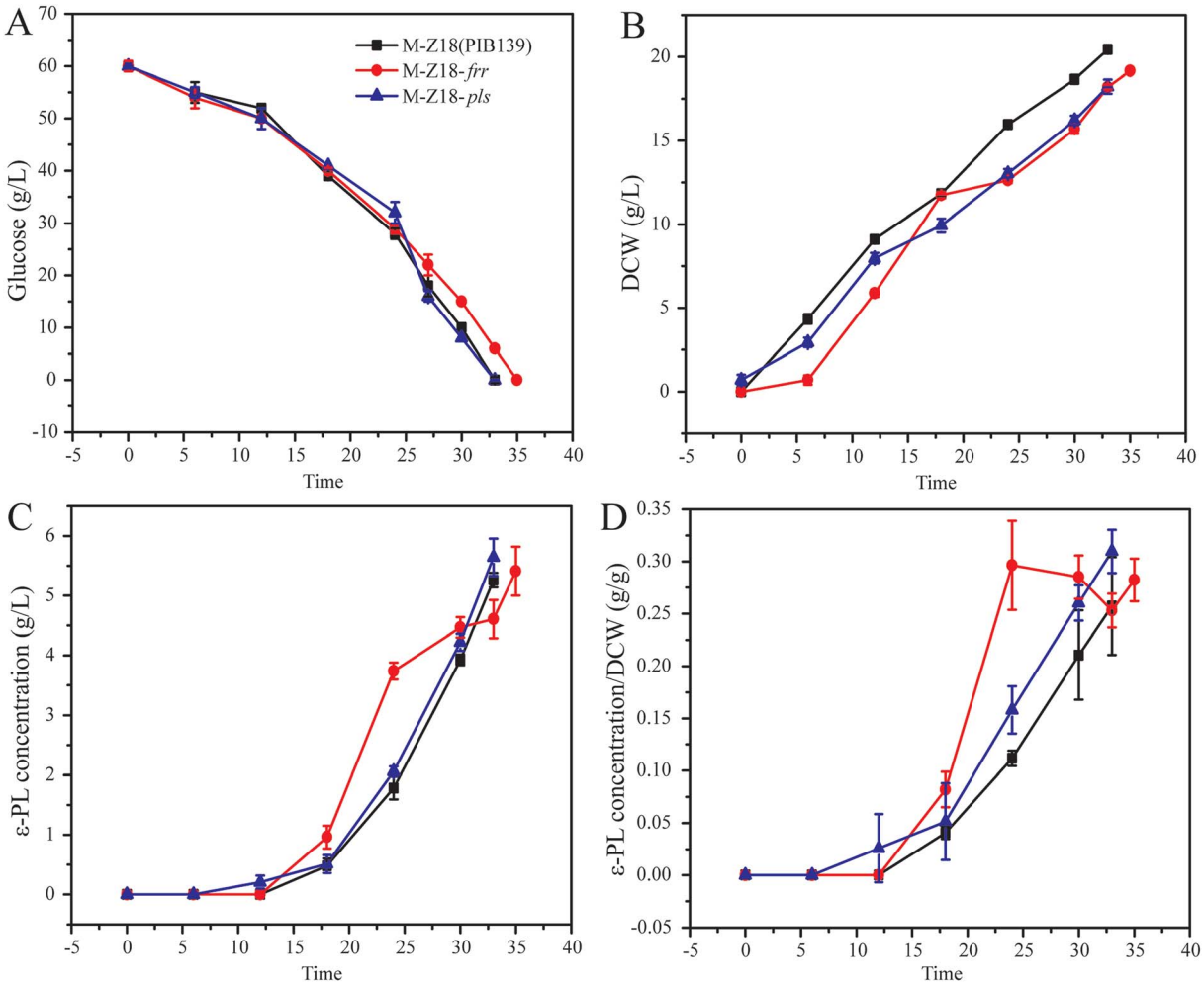

Fig. 6 Fermentation parameters of the parent strain *S. albulus* M-Z18 and overexpressed strain *S. albulus* M-Z18-*frr* and *S. albulus* M-Z18-*pls* in 1 L multi-bioreactor system.

Conclusions

In this study, iTRAQ-based proteomic analysis was used to explain the high-yield mechanism of streptomycin-resistant ε-PL-producing mutant in *Streptomyces albulus* at the level of proteomics. The dramatic changes were related to the carbohydrate metabolism, amino acid metabolism and energy metabolism based on the GO and KEGG analysis. Analysis of the proteome indicated that mutant SS-62 could not only strengthen the ε-PL precursor and energy metabolism but also tune the pathways related to transcriptional regulation and translation, resulting in a better intracellular metabolic environment for the synthesis of ε-PL. Specifically, the strain showed up-regulated expression levels of Pls and RRF in the pathway of L-lysine and ε-PL synthesis, which led to a significant increase of ε-PL biosynthesis capacity in the mutant strain.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Abbreviations

ε-PL	ε-Poly-L-lysine
iTRAQ	Isobaric tags for relative and absolute quantification
UniProt	Universal protein
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
qRT-PCR	Real time quantitative PCR
EMP	Embden-meyerhof-parnas
PPP	Pentose phosphate pathway
TCA	Tricarboxylic acid cycle
DAP	Diaminopimelate pathway
G6PDH	Glucose-6-phosphate dehydrogenase

PEPC	Phosphoenolpyruvate carboxylase
PYC	Pyruvate carboxylase
ASK	Aspartokinase
Pls	ε-PL synthetase
RRF	Ribosome recycling factor

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