SYSTEMS BIOTECHNOLOGY

SIMB
Society for Industrial Microbiology
and Biotechnology

Insights into the roles of exogenous glutamate and proline in improving streptolydigin production of *Streptomyces lydicus* with metabolomic analysis

Jing-Sheng Cheng · Shao-Fei Cui · Ming-Zhu Ding · Ying-Jin Yuan

Abstract The addition of precursors was one strategy to improve antibiotic production. The exogenous proline and glutamate, as precursors of streptolydigin, could significantly improve the streptolydigin production, but their underlying molecular mechanisms remain unknown. Herein, metabolomic analysis was carried out to explore the metabolic responses of *Streptomyces lydicus* to the additions of proline and glutamine. The significant differences in the quantified 53 metabolites after adding the exogenous proline and glutamate were enunciated by gas chromatography coupled to time-of-flight mass spectrometry. Among them, the levels of some fatty acids (e.g., dodecanoic acid, octadecanoic acid, hexadecanoic acid) were significantly decreased after adding glutamate and proline, indicating that the inhibition of fatty acid synthesis might be benefit for the accumulation of streptolydigin. Particularly, the dramatic changes of the identified metabolites, which are involved in glycolysis, the tricarboxylic acid cycle, and the amino acid and fatty acid metabolism, revealed that the additions of glutamate and proline possibly caused the metabolic cross-talk in *S. lydicus*. Additionally, the level of intracellular glutamate dramatically enhanced at 12 h after adding proline, showing that exogenous proline may be firstly convert into glutamate and consequently result in crease of the streptolydigin production. The high levels of streptolydigin at 12 and 24 h after adding glutamate unveiled that part glutamate were rapidly used to synthesize the streptolydigin. Furthermore, there is the significant difference in metabolomic characteristics of *S. lydicus* after adding glutamate and proline, uncovering that multiple regulatory pathways are involved in responses to the additions of exogenous glutamate and proline. Taken together, exogenous glutamate and proline not only directly provided the precursors of streptolydigin biosynthesis, but also might alter the metabolic homeostasis of *S. lydicus* E9 during improving the production of streptolydigin.

Keywords Glutamate · Proline · Metabolomic analysis · Streptolydigin · *Streptomyces lydicus*

J.-S. Cheng (✉) · S.-F. Cui · M.-Z. Ding · Y.-J. Yuan (✉)
Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, PO Box 6888, Tianjin 300072, People's Republic of China
e-mail: jscheng@tju.edu.cn

Y.-J. Yuan
e-mail: yjyuan@tju.edu.cn

J.-S. Cheng · S.-F. Cui · M.-Z. Ding · Y.-J. Yuan
Key Laboratory of Systems Bioengineering, School of Chemical Engineering and Technology, Tianjin University, Ministry of Education, PO Box 6888, Tianjin 300072, People's Republic of China

Introduction

Streptolydigin is a highly potential, broad-spectrum antibiotic with special structure characteristic that blocks the isomerization of RNA polymerase to an active configuration [30, 31]. Due to streptolydigin synthesis with a longest linear sequence of 24 steps starting from commercially available precursors [27], its chemical synthesis with the frequent use of organic reagents is horribly complex and causes environmental issues. Recently, strain improvement and fermentation optimization, two empirical approaches, have been utilized to improve the streptolydigin

🖄 Springer

productivity of *Streptomyces lydicus*. It has been reported that tailoring modification genes asparaginyl-tRNA synthetase-like *SlgZ*, methyltransferase *SlgM* and putative glycoside hydrolases genes *SlgC1*, *SlgC2* play significant roles in streptolydigin formation [15]. The overexpression of *SlgE3*, a gene responsible for the biosynthesis of glutamate in *S. lydicus*, can also result in an increase the yield of streptolydigin, while the expression of housekeeping glutamate synthases decreases when streptolydigin begins to be synthesized [14]. Additionally, the levels of glutamine synthetase I, glutamate synthase subunit beta, and glutamine synthetase are significantly down-regulated when the high pitching ratio increases the accumulation of streptolydigin in *S. lydicus* E9 [5]. Therefore, these works reveal that the metabolism of glutamate is closely associated with streptolydigin formation.

It has been reported that glutamate uptake is mediated by a glutamate-inducible, nonspecific transport system, whereas proline transport is regulated by at least two systems, and is not as stable as glutamate transport when the glutamate-inducible nonspecific system is utilized [24]. When antibiotic is not produced, the utilization of 2-oxoglutarate from the tricarboxylic acid cycle (TCA cycle) for producing the glutamate and C5 precursor is deceased in *S. clavuligerus* [18], while the pyruvate and alanine overflow point at an imbalance between carbon and nitrogen catabolism and biosynthetic fluxes in *S. lividans* TK24 [8]. Comparative LC–MS analysis demonstrates that the intermediates, which are involved in biosynthetic pathways of streptolydigin, are also significantly changed in the propionate-resistant mutant of *S. lydicus* [20]. Furthermore, the propionate supplement can result in an increase in streptolydigin production [21]. It has been reported that the intracellular levels of most amino acids (e.g., valine, alanine, glutamate, and proline) are decreased with a significant increase of streptolydigin in *S. lydicus* E9 under high pitching ratio [6]. The depletion of glutamate and aspartate has been confirmed to lead to a distinct shift in fluxes of the central carbon and nitrogen metabolism [9]. Although the additions of exogenous glutamate and proline can dramatically enhance the streptolydigin production in *S. lydicus* E9, it is still unclear how the exogenous glutamate and proline impact the primary and secondary metabolism of *S. lydicus* during streptolydigin production.

In this study, the metabolomic profiling of *S. lydicus* E9 after adding glutamate and proline (100 mg l^{-1}) were employed with gas chromatography coupled to time-of-flight mass spectrometry (GC–TOF–MS). The significant differences in the streptolydigin levels and metabolic characteristics of *S. lydicus* E9 were observed after the additions of exogenous glutamate and proline, respectively. This investigation will help us to comprehensively elucidate the roles of the additions of exogenous glutamate and proline when improving the streptolydigin production in a global metabolic view.

Materials and methods

Strain and cultivation conditions

The strain of *S. lydicus* E9 (CGMCC NO.3075), which produces the streptolydigin, was used in this work. Firstly, *S. lydicus* E9 was cultured in solid culture medium for approximate 7 days. Afterwards, strains from the solid medium were inoculated into a 250-mL flask containing 50 ml of seed medium for 48 h as the first-grade seeds. Subsequently, 10 ml of first-grade seeds was transferred into another 90-ml seed medium and cultivated about 36 h as second-grade seeds [5, 6]. The production of streptolydigin was carried out in fermentors (Baoxin Biotech Co., LTD, Shanghai, China) containing 3.0 l medium (40.0 g l^{-1} starch, 5.0 g l^{-1} glucose, 2.0 g l^{-1} peptone, 1.0 g l^{-1} K$_2$HPO$_4$, 0.5 g l^{-1} Mg$_2$SO$_4$·7H$_2$O, and 0.5 g l^{-1} NaCl) at 28 ± 0.5 °C with stirring rate fixed to 500 rpm and pH adjusted to 6.5 HCl (3.0 mol l^{-1}) and NaOH (3.0 mol l^{-1}) automatically.

Additions of glutamate and proline

The glutamate and proline (final concentration 100 mg l^{-1}) were added into the media at the beginning of fermentation, respectively. The glutamate and proline were firstly dissolved in Milli-Q water and then filtered through a 0.22-µm filter before adding into the medium [6].

Measurement of glucose and biomass

The supernatant of 5-ml broth at the pre-determined time was collected after centrifuging for 5 min at 12,000 rpm. Residual sugar was measured by the biosensor (SBA-40E, Shandong, China). In addition, the cell pellet of 40-ml broth was washed three times with Milli-Q water after centrifuging at 4,500 rpm for 10 min, and then dried at 85 °C for 15 h to obtain the dry cell weight (DCW).

Analysis of streptolydigin level

The streptolydigin concentration in the fermentation broth was detected according to the method of Li et al. [20]. The streptolydigin was measured by HPLC analysis with the C$_{18}$ reversed-phase (250 × 4.6 mm; 5-µm particle size) column (Thermo Electron Corp., Waltham, MA, USA) and a 330-nm ultraviolet detector. The ammonium acetate (0.01 mol l^{-1}) in methanol containing 35 % water was

used as a mobile phase with a flow rate of 0.6 ml min^{-1} at room temperature.

Intracellular metabolite extraction

The intracellular metabolites of S. lydicus E9 at 12, 24, and 72 h after adding glutamate and proline were extracted and derivatized as described previously [10]. Cell pellets were collected and ground to a fine powder in liquid nitrogen after quenching by 60 % methanol (-40 °C, v/v) and washing with deionized water. Fresh cells (50 mg) were extracted twice by methanol/water (1:1, v/v, -40 °C). After combining the two supernatants, 10 μl succinic d4 acid (0.14 mg ml^{-1}) was added as internal standard to aliquots of 100 μl before lyophilization. For chemical derivatization, the dried extract was firstly dissolved in the 50 μl of methoxamine hydrochloride in pyridine (20 mg ml^{-1}) at 40 °C for 80 min. Consequently, for trimethylsilylation, 80 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and re-incubated at 40 °C for 80 min.

Identification of intracellular metabolites with gas chromatography-time of flight–mass spectrometry (GC–TOF–MS)

After the metabolites derivatized, these samples were analyzed by GC–TOF–MS as described previously [10]. A 1-μl sample was injected by an Agilent 7683 auto-sampler into Agilent 6890 GC, which was equipped with a fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm DB-5MS, J&W Scientific, Folsom, CA) with the following temperature program: after a 2-min delay at 70 °C, the oven temperature increased to 290 °C at the rate of 5 °C min^{-1}, holding for 3 min. Metabolites were identified by comparing their mass fragmentation with the NIST library and Golm Metabolome database. All the peak areas were normalized against the internal standard for further processing. Pattern recognition methods were employed to analyze the data sets from metabolite profiles. The datasets derived from GC–TOF–MS were mean-centered and pareto-scaled before performing principal components analysis (PCA) using SIMCA-P 11.5 Demo software. Five replicates were used to perform multivariate analysis for each sample.

Results

Changes in metabolomic profiles of S. lydicus after adding exogenous glutamate and proline

Previous work displayed that the levels of intracellular glutamate and proline decreased significantly with the

dramatic increases of streptolydigin production under the high pitching ratio [6]. As displayed in Fig. 1a, although the additions of exogenous glutamate and proline (100 mg l^{-1}) both significantly enhanced the accumulation of streptolydigin, the levels of streptolydigin before 72 h were increased faster by exogenous glutamate than by proline (Fig. 1a). Particularly, the streptolydigin level remarkably increased at 12 h after adding exogenous glutamate, while its level was no obvious change after adding proline. Furthermore, although the proline addition did not significantly alter the streptolydigin level at 24 h, there was significantly different in the cellular growth between the control and the additions of glutamate and proline (Fig. 1c). Thus, to investigate the potential mechanism of the glutamate and proline addition how to impact the cellular growth and streptolydigin titer, we chose the 12-, 24-, and 72-h samples for the detailed metabolomic analysis by GC–TOF–MS.

Figure 2 illustrates the changes in metabolomic profiling of S. lydicus E9 at 12, 24, and 72 h after adding the exogenous glutamate and proline. A total of 53 significantly changed metabolites were quantified by GC–TOF–MS. Most metabolites were mainly involved in the carbohydrates (e.g., glucose and fructose), amino acids (e.g., valine, and leucine), organic acids (e.g., succinic acid, and pyruvic acid), polyols (e.g., inositol), alkane (e.g., tetradecane, pentadecane, and hexadecane) and fatty acids (e.g., dodecanoic acid, octadecanoic acid, and hexadecanoic acid). These results displayed that there were significant differences in metabolic profiling among the control cells and the cells after adding exogenous glutamate and proline.

Principal component analysis (PCA) of the identified metabolites in S. lydicus E9

PCA was employed to evaluate the variations of the metabolic levels of S. lydicus E9 at 12, 24, and 72 h after treatment with glutamate and proline. As shown in the score plot (Fig. 3a1, a2, b1, b2), the samples from the glutamate- and proline-treated S. lydicus E9 at 12 and 72 h were clustered clearly, however, the samples from the fermentation 24 h were obviously scattered (Fig. S1). These results further verified that exogenous glutamate and proline produced the global changes in the metabolic characteristics of S. lydicus.

Meanwhile, the loading plots were employed to detect the potential roles of the identified metabolites in influencing the streptolydigin production after adding the glutamate and proline. Each point in the loading plot indicates a mass fragment of a certain metabolite. The further a data point is from the origin, the greater the component belongs to influence the cluster formation [5]. As illustrated in
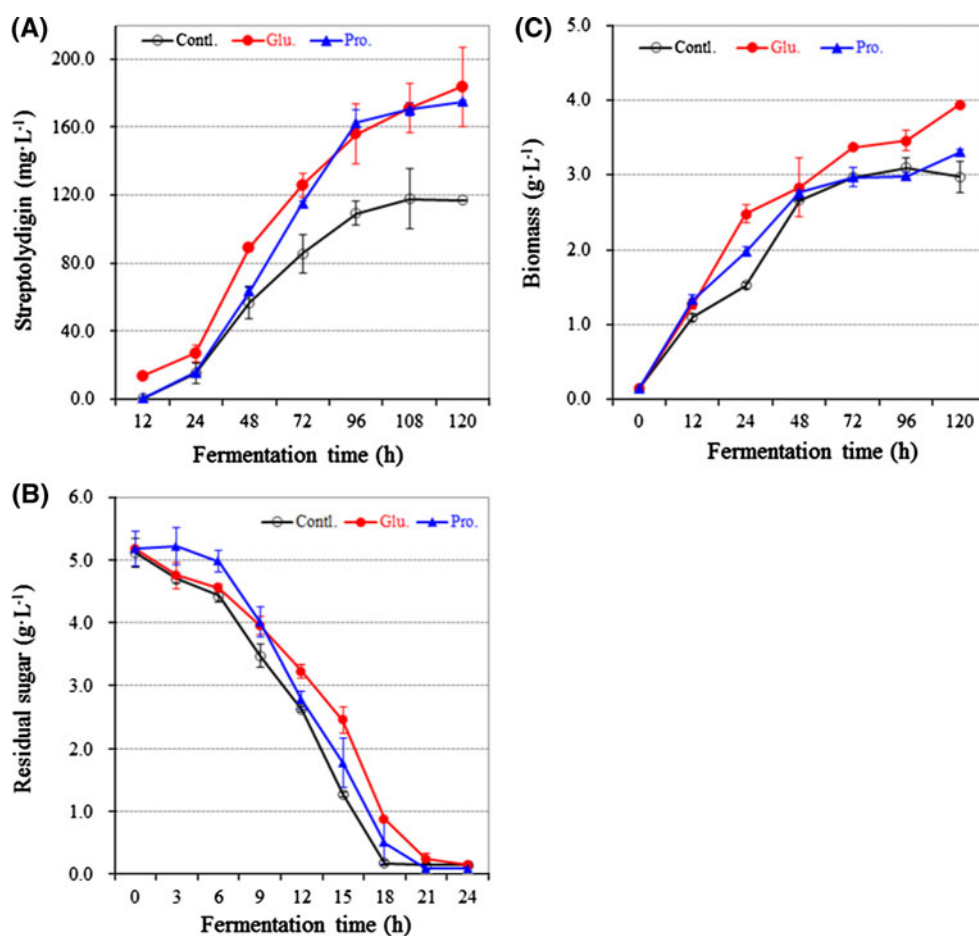
Fig. 1 Alterations in streptolydigin production (**a**) and residual sugar (**b**) of *S. lydicus* E9 after the additions of glutamic acid and proline (100 g l^{-1}). The samples were collected to detect the variations in metabolomic profile of *S. lydicus* E9 at pre-determined times of 12, 24, and 72 h, respectively

Fig. 3, a total of 11 compounds were detected to be potential biomarkers for distinguishing the samples at 12 and 72 h after treatment with the exogenous glutamate and proline. Among them, seven compounds (including alanine, glycine, valine, cAMP, glucose, hexadecanoic acid, and octadecanoic acid) were considered as potential biomarkers for distinguishing the samples at both 12 and 72 h. However, the lactic acid and ethanedioic acid were only as biomarkers for telling the samples at 12 h. The succinic acid and glycerol were only as biomarkers for distinguishing the samples at 72 h (Fig. S2).

Impacts of exogenous glutamate and proline on the amino acid metabolism of *S. lydicus* E9

As displayed in Figs. 2 and 4, a total of 11 amino acids were significantly changed after treatment with glutamate and proline. At 12 h, the exogenous glutamate dramatically decreased the intracellular levels of tyrosine, lysine, threonine, glycine, leucine, and valine, whereas the exogenous proline resulted in the decrease in the level of serine and the increases in the levels of lysine, glutamine, proline, and valine. Similar results were found at 24 and 72 h after adding glutamate and proline. Especially the addition of proline significantly enhanced the intracellular level of glutamate, which reached 5.9-fold as compared to that in the control at 12 h, indicating that some exogenous proline was quickly converted into glutamate. Although the intracellular level of glutamate was also increased (3.5-fold) as compared to that in the control at 12 h, it was likely that the most exogenous glutamate was transported into cells. However, the intracellular levels of glutamate dramatically reduced to 0.37- and 0.48-fold at 24 h after adding glutamate and proline as compared to the control, respectively (Fig. 4b). Meanwhile, the levels of valine, leucine, and glycine were significantly increased at 72 h after adding proline (Fig. 4c). Particularly, the intracellular levels of proline always maintained around 2-fold as compared to that in the control whether at 12, 24, or 72 h after adding exogenous proline. The intracellular levels of proline at 12

Fig. 2 Heat map representation of the identified metabolites by GC–TOF–MS and clustering of the identified metabolites data by CLICK (CLuster Identification via Connectivity Kernels) in Expander 4.1 software. Shades of *red* and *green* indicate the increase and decrease of metabolite levels, respectively
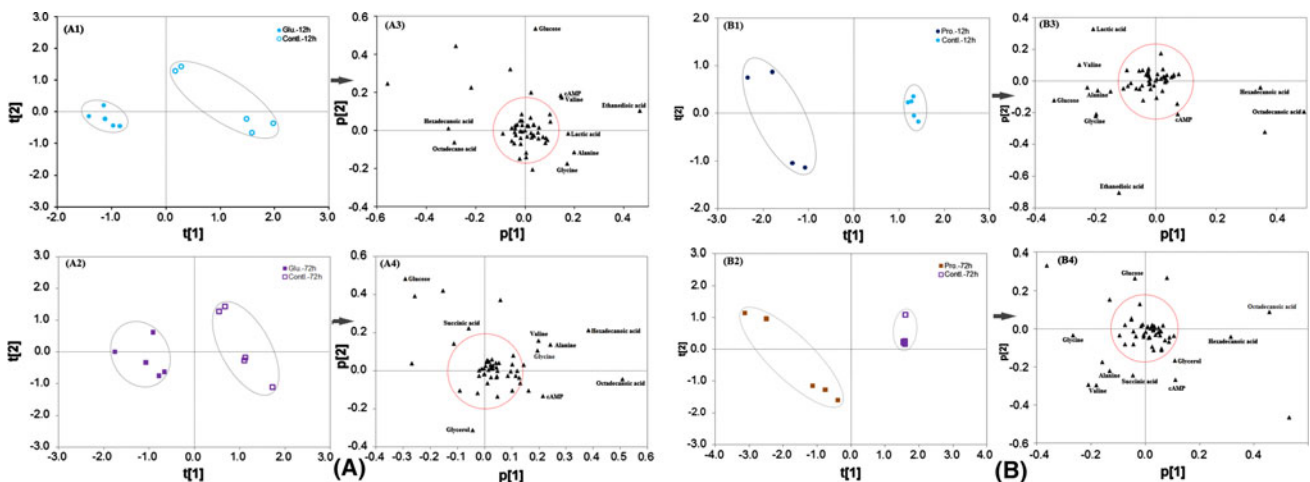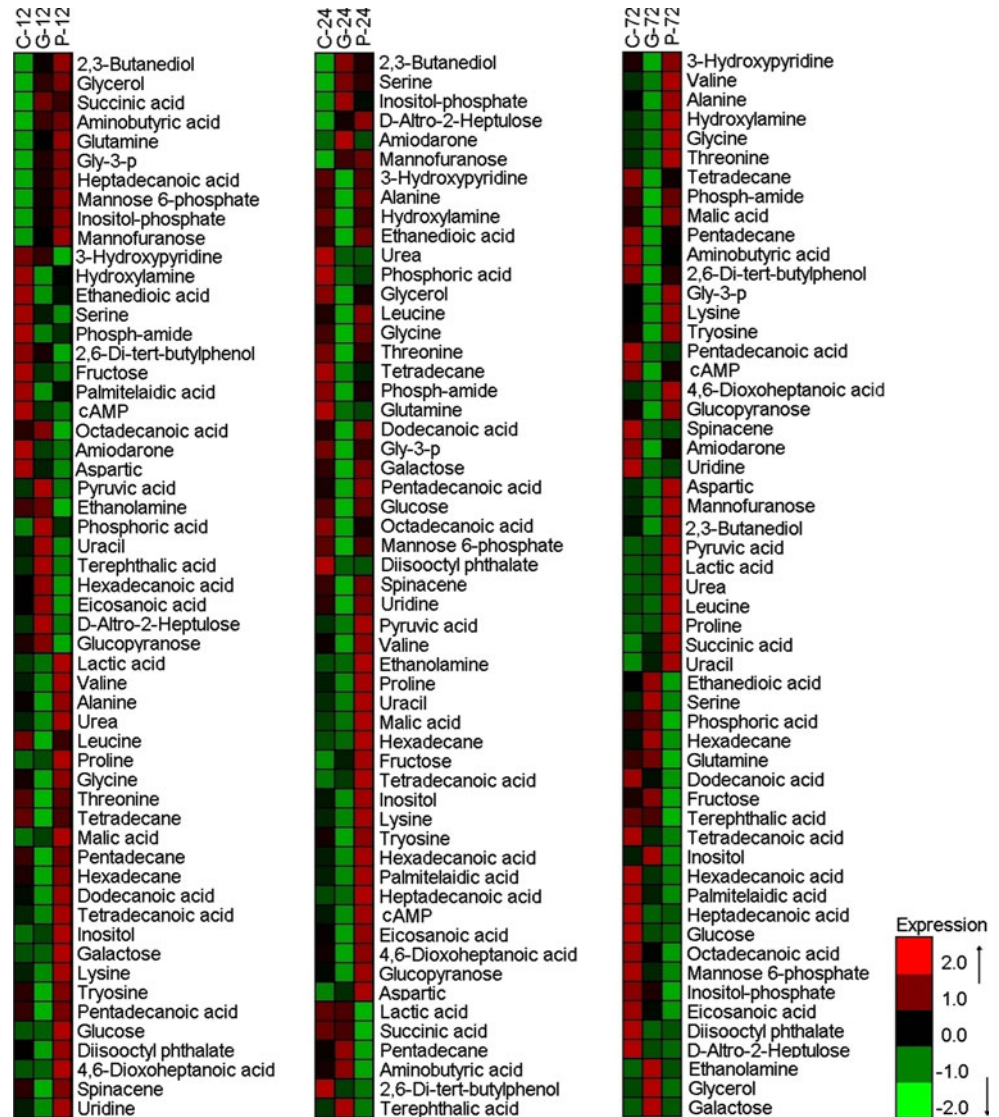

Fig. 3 PCA score plot of metabolites of *S. lydicus* E9 after treatment with exogenous glutamate (**a**) and proline (**b**). *a1*, *a2* Score of the samples at 12 and 72 h after treatment with glutamate; *b1*, *b2* score of the samples at 12 and 72 h after treatment with proline; *a3*, *a4*, b3, b4 the OPLS loading plot of samples tested at 12 and 72 h after treatment with glutamate and proline, respectively

and 72 h were no significant changes, while their levels were dramatically decreased at 24 h after adding exogenous glutamate. These results reveal that the additions of exogenous glutamate and proline directly or indirectly alter the amino acid mechanism during improving the accumulation of streptolydigin.

Variations of the levels of fatty acids in *S. lydicus* E9 after adding exogenous glutamate and proline

Figure 5 shows that the relative levels of dodecanoic acid, eicosanoic acid, tetradecanoic acid, and octadecanoic acid at 12 and 72 h were much higher than that at 24 h. However, the levels of palmitelaidic acid and heptadecanoic acid increased significantly after 12 h whether in the control cells or in the cells after treatment with glutamate and proline. Meanwhile, the levels of dodecanoic acid, eicosanoic acid, pentadecanoic acid, and palmitelaidic acid maintained rather low around 0.04. However, the levels of octadecanoic acid and hexadecanoic acid were rather high (Fig. 5g, h), which is a possible reason why they are potential biomarkers with PCA.

Changes of the levels of polyols and polyamines in *S. lydicus* E9 after adding exogenous glutamate and proline

We also observed that the relative abundance of hydroxylamine at 24 h was higher than that at 12 and 72 h after adding glutamate and proline, while the changes in the phosphamide levels were opposite in the control cells and the cells after treatment with the glutamate and proline (Fig. 6). It was interesting that the levels of ethanolamine at 72 h were much higher than that at 12 and 24 h whether in the control or in the cells treated with glutamate and proline (Fig. 6). Furthermore, we also detected polyols including 2,3-butanediol, inositol, and inositol-phosphate. Among them, the levels of 2,3-butanediol at 24 h were higher than that at 12 and 72 h. The levels of inositol-phosphate were gradually increased with the fermentation progress. The changing trends of the glycerol levels at 72 h after adding glutamate and proline were similar with that of the streptolydigin accumulation.

Influences of exogenous glutamate and proline on the intermediates of the central carbon metabolism

As illustrated in Fig. 7, the identified potential biomarkers, including glucose, valine, glycine, alanine, glycerol, lactic acid, and succinic acid, were closely associated with the central carbon metabolic pathway. The addition of proline significantly elevated the levels of valine and glycine, which are derived from the glycolysis intermediates

pyruvate and 3-phosphoglycerate (3-PGA), respectively. However, the levels of valine and glycine under glutamate treatment were opposite. The addition of proline caused the accumulation of the intracellular glucose at 12 h, while the
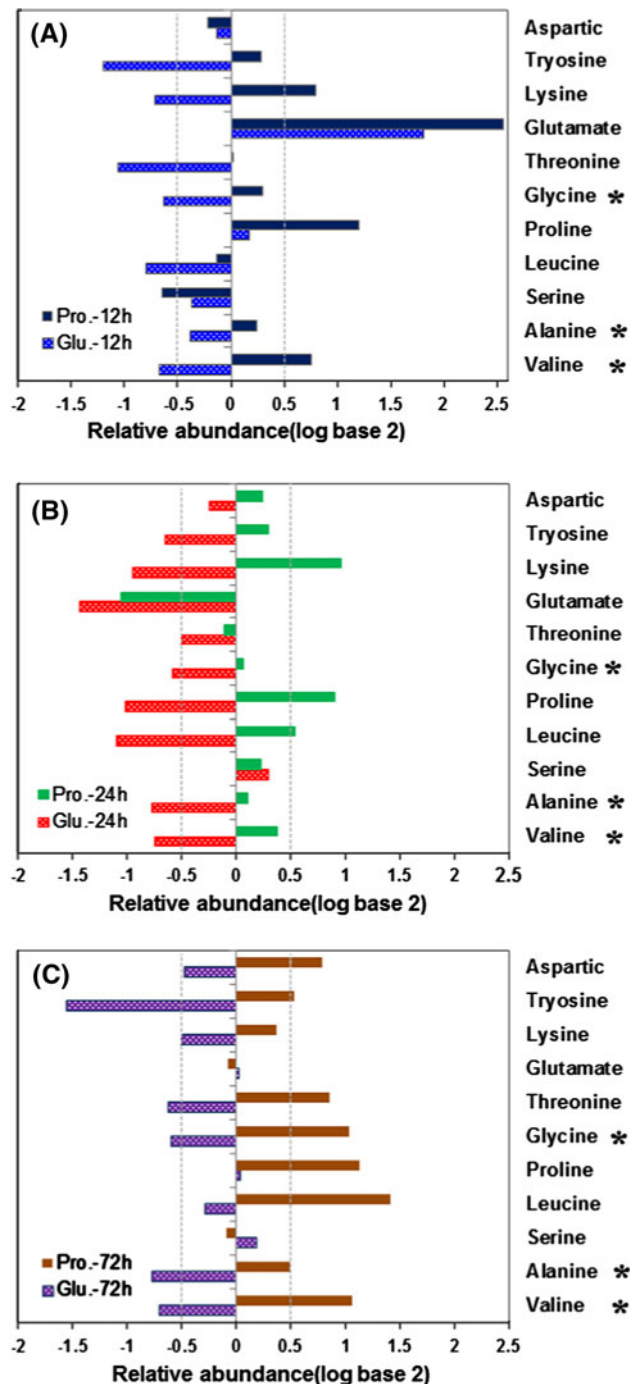

Fig. 4 The relative alterations of amino acids levels in *S. lydicus* E9 at 12, 24, and 72 h after treatment with glutamate and proline, respectively. The variation folds of metabolites were obtained by normalizing peak area of each metabolite in samples, and then the logarithmic value (base 2) was calculated for the mean value of five replicates. *Asterisk* indicates the potential biomarkers after PCA
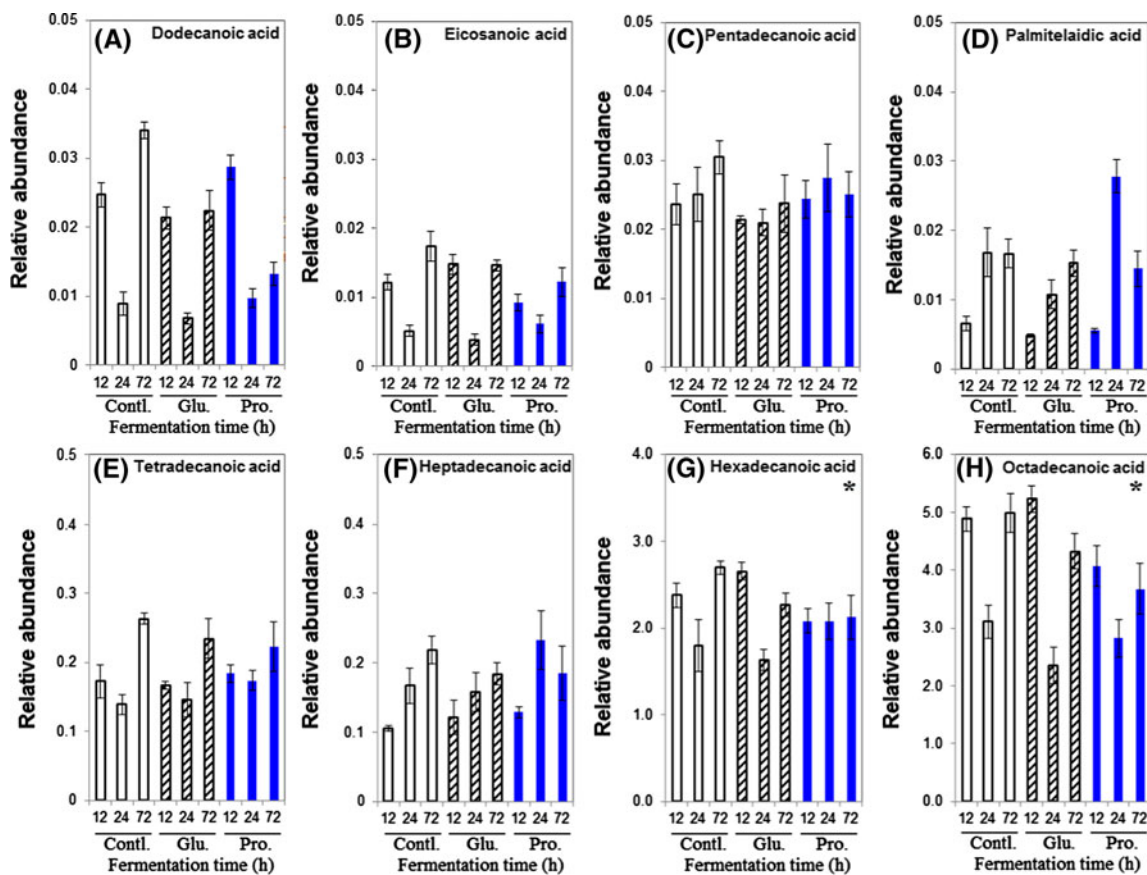
Fig. 5 Variations of intracellular fatty acids in *S. lydicus* E9 significantly changed after treatment with glutamate and proline, respectively. The relative abundance was calculated by normalization of peak area of each metabolite to internal standard ribitol, and each value represents the mean of five independent replicates ± SD. *Asterisk* indicates the potential biomarkers after PCA

intracellular levels of glucose at 72 h after adding the glutamate and the control were higher than that at 12 and 24 h, indicating that the exogenous proline accelerated the glucose utilization. Meanwhile, the levels of 3-PGA at 24 h were higher than that at 12 and 72 h after adding glutamate and proline. It was also found that pyruvate rapidly decreased at 12 h after adding the glutamate, while its levels significantly enhanced after adding proline. These trends suggest that the additions of exogenous glutamate and proline alter the levels of intermediates of the central carbon and amino acid metabolism, and consequently improve the streptolydigin biosynthesis.

Discussion

In this work, we investigated metabolomic profiling in *S. lydicus* E9 after adding the glutamate and proline, so as to better understand amino acid utilization and how to manipulate it. Our results revealed that the additions of glutamate and proline led to significant variations of metabolites involved in carbohydrates, amino acids, organic acids, and fatty acids metabolism (Figs. 2, 7).

Furthermore, the exogenous glutamate and proline not only improved the accumulation of streptolydigin but also altered cellular growth, especially the addition of glutamate causing the increases in biomass (Fig. 1). The novel links between antibiotic production and primary metabolism have been uncovered in *S. coelicolor* by individual mispredictions and the stoichiometric matrix, [2]. It has been reported that the significant changes in the intermediates of glycolysis and TCA cycle, amino acids, and polyamines can partly explain the differences of primary and secondary metabolism growth between pilot and industrial processes [11]. The flux increase and the intermediate accumulation of TCA cycle alter the regulation of the secondary metabolite biosynthesis [16, 28]. Therefore, these results indicate that the additions of exogenous glutamate and proline give rise to the shifts between primary and secondary metabolism and consequently influences the streptolydigin biosynthesis and cellular growth of *S. lydicus* E9 under the different stages of fermentation.

Streptomyces antibiotic possesses an energy-dependent, carrier-mediated transport system for the uptake of glutamate and proline. Our experimental data displayed that the intracellular glutamate levels dramatically increased at 12 h
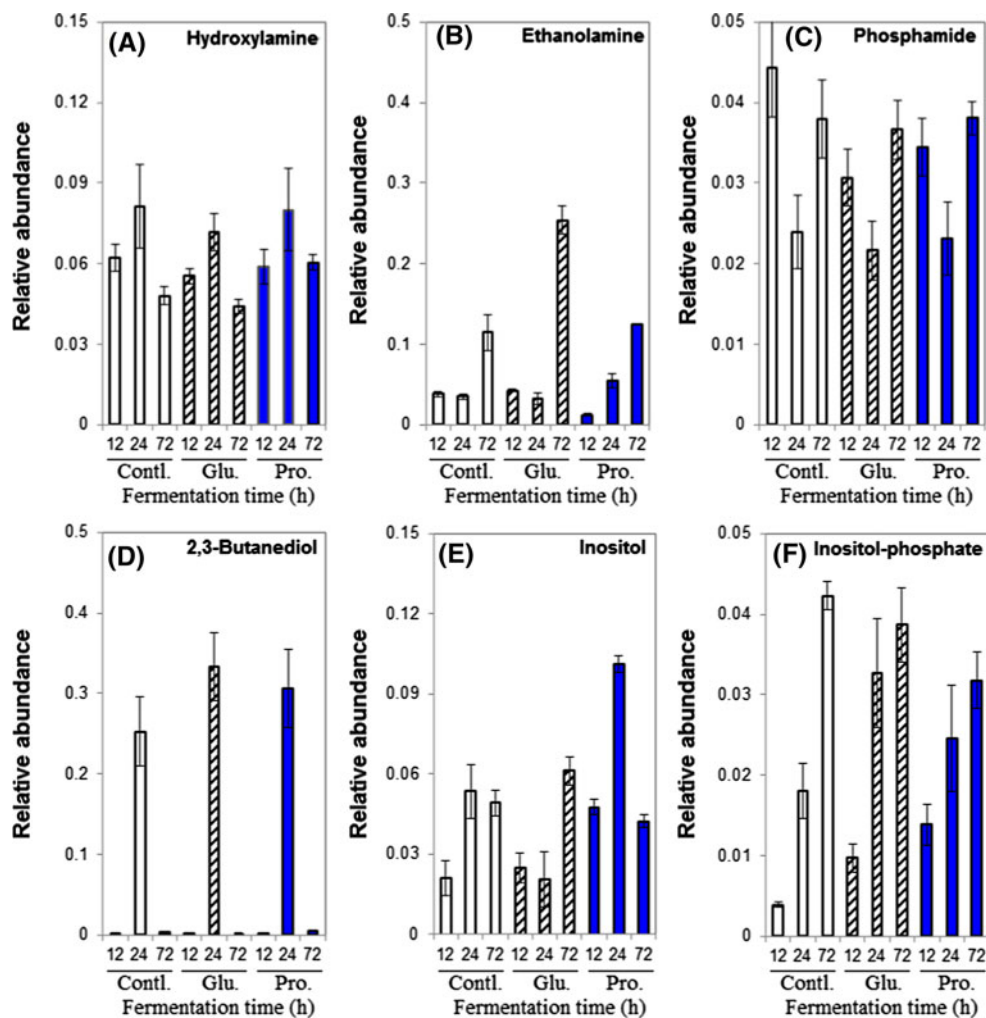
Fig. 6 Changes of intracellular amine and polyol in *S. lydicus* E9 related to after treatment with glutamate and proline, respectively. The relative abundance was calculated by normalization of peak area of each metabolite to internal standard ribitol, and each value represents the mean of five independent replicates ± SD

after additions of glutamate and proline (Figs. 4, 7), indicating that exogenous glutamate rapidly transports into cells. However, the level of intracellular glutamate in the cells after adding glutamate was lower than that in the cells treated with proline (Fig. 4), revealing that the proline may be promptly converted into glutamate, since proline can be metabolized to Δ1-pyrroline-5-carboxylate by proline dehydrogenase and then converted into glutamate [4]. In *S. coelicolor*, the addition of proline is able to improve the activity of proline catabolic enzymes [29]. It has been demonstrated that glutamate uptake is regulated by a glutamate-inducible, non-specific transport system, while proline transport is regulated by at least two systems and is not as stable as glutamate transport [24]. Glutamic acid is most favorable to the formation of streptolydigin [21]. Thus, these results possibly explain why the streptolydigin accumulates so quickly at 12 and 24 h after adding exogenous glutamate, while there is no significant change after adding proline. As compared to the

control, the intracellular levels of proline remarkable increased and kept as much as twofold of the control. Based on the above analysis, these results indicate that there exist different responsive and regulatory mechanisms to the additions of exogenous glutamate and proline in *S. lydicus* E9.

The central carbon metabolism can provide a sufficient supply of carbon source for efficiently producing secondary metabolites. In our experiment, whether at 12, 24, or at

Fig. 7 Changes in the relative levels of metabolites involved in glycolysis, TCA cycle, and amino acids biosynthesis in *S. lydicus* E9 after treatment with glutamate and proline, respectively. The relative abundance was calculated by normalization of peak area of each metabolite to internal standard ribitol, and each value represents the mean of five independent replicates ± SD. The *asterisk* indicates the potential biomarkers after PCA. The *X-axis* denotes the fermentation time of the control group and the glutamate and proline treatment groups, and the *Y-axis* denotes relative abundance of the detected metabolites with GC–TOF–MS after applying volume normalization
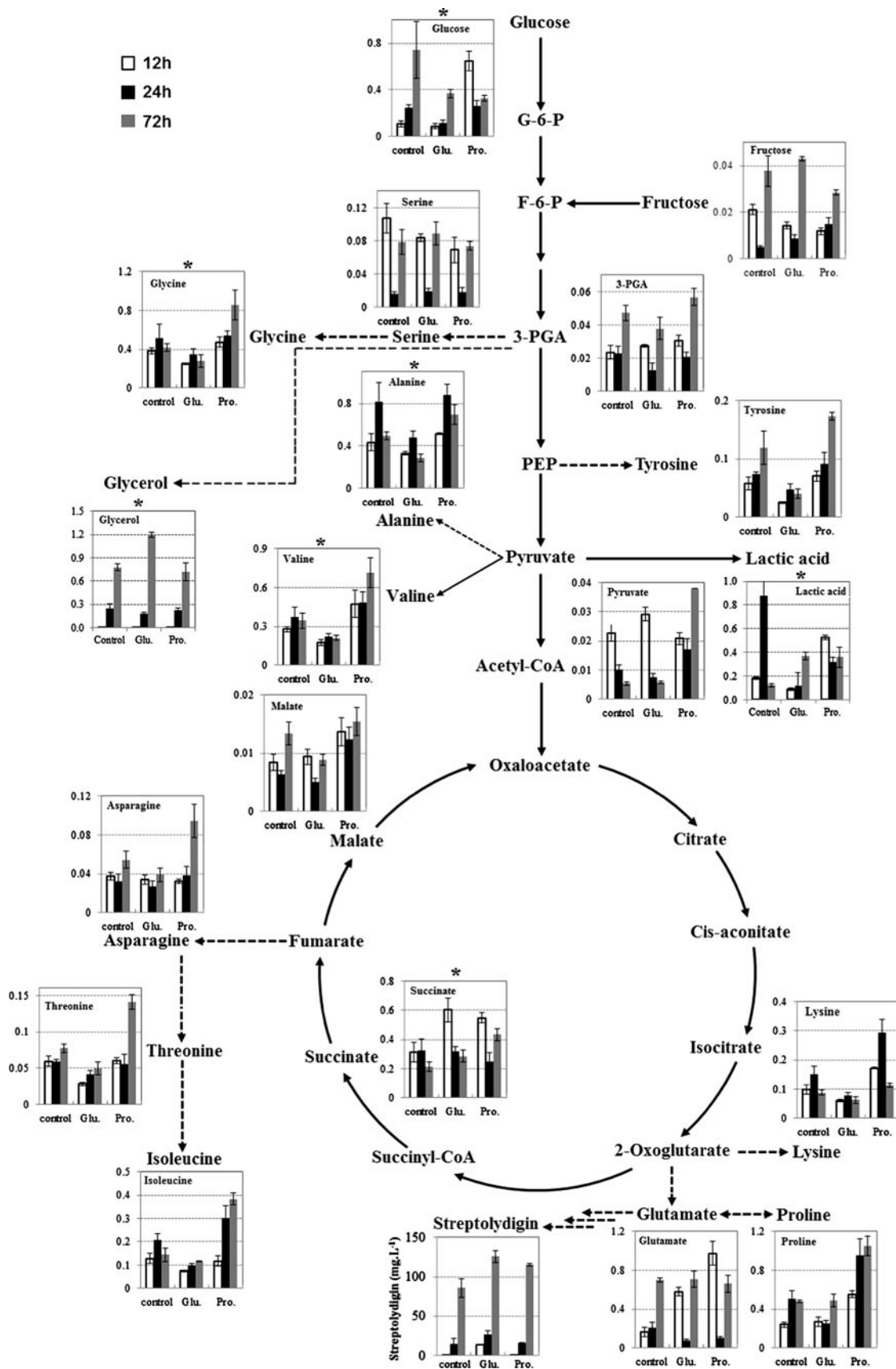
72 h, although both exogenous glutamate and proline stimulated the streptolydigin production, the levels of alanine, valine, and glycine, as potential biomarkers, were much lower after adding glutamate than that after adding proline and in the control cells (Figs. 7, S2). It has been demonstrated that glutamate and alanine can be used as both a nitrogen and carbon source to produce pristinamycin, while valine can only be utilized as a nitrogen source in *S. pristinaespiralis* [32]. When glutamate was employed as a nitrogen source, the alanine dehydrogenase was up-regulated in *S. coelicolor* [12]. The depletion of glutamate and aspartate causes a distinct shift in fluxes of the central carbon and nitrogen metabolism [9]. Therefore, this work presents that the glutamate addition likely accelerates the conversions of alanine, valine, and glycine or inhibits their biosynthesis. Moreover, the additions of exogenous glutamate and proline altered the level of intracellular glucose (Fig. S2). Taken together, these results reveal that the additions of exogenous glutamate and proline change the carbon source for the cellular growth and streptolydigin synthesis of *S. lydicus* E9.

Furthermore, the glycolysis pathway and TCA cycle are able to supply energy and the sufficient precursors including amino acids for secondary metabolites biosynthesis. The glutamic acid is taken as amino donor for the formation of *p*-aminobenzoic acid, as a precursor of candicidin in *S. griseus* [13]. It has been reported that glutamate and proline as precursors were directly involved in the streptolydigin biosynthesis of *S. lydicus* [6, 26]. The glutamate in *S. lydicus* was mainly synthesized by glutamine and 2-oxoglutarate and catalyzed by combined enzyme agent SlgE1–SlgE2–SlgE3 [14, 25]. Herein, the addition of glutamate dramatically decreased the levels of most amino acids, while the addition of proline elevated the levels of the amino acids, especially at 72 h (Fig. 4). So, the exogenous glutamate and proline directly or indirectly alter the amino acid mechanism during improving the accumulation of streptolydigin. In *S. lydicus* E9, the remarkable increase in streptolydigin accompanies with the decreases in the intracellular levels of most amino acids under high pitching ratio [6]. Metabolomic analysis also reveals that the salt stress up-regulates a large fraction of amino acids [19]. Moreover, in this work, the levels of streptolydigin and intracellular glycerol had the same change trends (Fig. 7). Glycerol-3-phosphate (Gly-3-P), an important part of glycerol metabolism, is readily formed from glycerol by ATP-dependent phosphorylation catalyzed by glycerol kinas. We observed that the levels of Gly-3-P in *S. lydicus* E9 at 24 and 72 h after adding glutamate were much lower than that after adding proline and in the control cells (Fig. 2). The energy-producing reaction of the 2-oxoglutarate degradation competes with the glutamate synthesis via nitrogen incorporation into 2-oxoglutarate [3]. In *S. ambofaciens*, the increases in the initial concentrations of glycerol

cause an enhancement in the specific growth rate with a drop in spiramycin production when it cultivated with valine as a nitrogen source [23]. So, the additions of glutamate and proline change the status of energy in *S. lydicus* E9, which may influence the streptolydigin biosynthesis.

Additionally, as a potential biomarker, the cAMP levels at 24 h after adding glutamate were lower than that in the control and after adding proline (Figs. 2, S2). Interestingly, whether in the control or in the cells treated with glutamate or proline, the cAMP levels at 24 h were significantly lower than that at 12 and 72 h (Fig. 2). The intracellular cAMP maintained a lower level under 30 % pitching ratio with a higher streptolydigin production [6]. The cAMP has been reported to be involved in regulating the biosynthesis of the antibiotic and morphological differentiation of the mycelium [22]. Thus, the changes of cAMP levels are likely involved in regulating streptolydigin formation.

The inhibition of a target in fatty acid biosynthesis can give rise to a different phenotype, which enhances the understanding of secondary metabolism and the life cycle of the streptomycete [1]. In this work, the intracellular levels of some fatty acids (e.g., dodecanoic acid, octadecanoic acid, hexadecanoic acid) were significantly decreased after the addition of glutamate and proline (Fig. 5). It has been confirmed that pH shock particularly promotes fatty acid degradation and suppressed the fatty acid biosynthesis when it positively induces the actinorhodin biosynthesis [17]. The present work also displays that SlyfabCF is shared by fatty acid and streptolydigin synthesis [33]. Small molecules remodel the yields of secondary metabolites by inhibiting fatty acid biosynthesis in many actinomycetes [7]. Our experiment data herein show that the exogenous glutamate and proline likely inhibit the synthesis of fatty acids and consequently result in the accumulation of streptolydigin in *S. lydicus* E9. Taken together, the additions of exogenous glutamate and proline not only directly provide precursors of streptolydigin synthesis but also possibly alter the metabolic homeostasis of carbon, nitrogen, and energy. These metabolic variations are very rapid, specific, and widespread across metabolism during improving streptolydigin biosynthesis.

Conclusions

The significant changes of the identified metabolites are involved in the glycolysis, tricarboxylic acid cycle, amino acids, and fatty acids metabolism after adding exogenous glutamate and proline. The difference in metabolomic characteristics of *S. lydicus* after treatment with exogenous glutamate proline uncovered that there are different responsive and regulatory mechanisms to exogenous glutamate and proline. Meanwhile, the inhibition of the fatty

acid synthesis and changes in glycerol and Gly-3-p indicate that exogenous glutamate proline alter the energy metabolism in *S. lydicus* E9. The variations of cellular growth and streptolydigin uncover that the additions of exogenous glutamate and proline give rise to the shifts between primary and secondary metabolism. So, this work reveals that exogenous glutamate and proline not only directly provide precursors of streptolydigin synthesis but also likely alter the metabolic homeostasis so as to improve the streptolydigin production of *S. lydicus* E9.

References

1. Ahmed S, Craney A, Pimentel-Elardo SM, Nodwell JR (2013) A synthetic, species-specific activator of secondary metabolism and sporulation in *Streptomyces coelicolor*. Chem Bio Chem 14(1):83–91

2. Alam MT, Merlo ME, STREAM Consortium, Hodgson DA, Wellington EM, Takano E, Breitling R (2010) Metabolic modeling and analysis of the metabolic switch in *Streptomyces coelicolor*. BMC Genomics 11:202

3. Araújo WL, Trofimova L, Mkrtchyan G, Steinhauser D, Krall L, Graf A, Fernie AR, Bunik VI (2013) On the role of the mitochondrial 2-oxoglutarate dehydrogenase complex in amino acid metabolism. Amino Acids 44(2):683–700

4. Berney M, Weimar MR, Heikal A, Cook GM (2012) Regulation of proline metabolism in mycobacteria and its role in carbon metabolism under hypoxia. Mol Microbiol 84:664–681

5. Cheng JS, Lv XM, Yuan YJ (2012) Investigation of proteomic responses of *Streptomyces lydicus* to pitching ratios for improving streptolydigin production. Biotechnol Bioproc E 17:997–1007

6. Cheng JS, Liang YQ, Ding MZ, Cui SF, Lv XM, Yuan YJ (2013) Metabolic analysis reveals the amino acid responses of *Streptomyces lydicus* to pitching ratios during improving streptolydigin production. Appl Microbiol Biotechnol. doi:10.1007/s00253-013-4790-4

7. Craney A, Ozimok C, Pimentel-Elardo SM, Capretta A, Nodwell JR (2012) Chemical perturbation of secondary metabolism demonstrates important links to primary metabolism. Chem Biol 19(8):1020–1027

8. D'Huys PJ, Lule I, Van Hove S, Vercammen D, Wouters C, Bernaerts K, Anné J, Van Impe JF (2011) Amino acid uptake profiling of wild type and recombinant *Streptomyces lividans* TK24 batch fermentations. J Biotechnol 152(4):132–143

9. D'Huys PJ, Lule I, Vercammen D, Anné J, Van Impe JF, Bernaerts K (2012) Genome-scale metabolic flux analysis of Streptomyces lividans growing on a complex medium. J Biotechnol 161(1):1–13

10. Ding MZ, Cheng JS, Xiao WH, Qiao B, Yuan YJ (2009) Comparative metabolomic analysis on industrial continuous and batch ethanol fermentation processes by GC–TOF–MS. Metabolomics 5:229–238

11. Ding MZ, Lu H, Cheng JS, Chen Y, Jiang J, Qiao B, Li BZ, Yuan YJ (2012) Comparative metabolomic study of *Penicillium chrysogenum* during pilot and industrial penicillin fermentations. Appl Biochem Biotechnol 168(5):1223–1238

12. Fisher SH (1989) Glutamate synthesis in *Streptomyces coelicolor*. J Bacteriol 171(5):2372–2377

13. Gil JA, Naharro G, Villanueva JR, Martin JF (1985) Characterization and regulation of *p*-aminobenzoic acid synthase from *Streptomyces griseus*. J Gen Microbiol 131:1279–1287

14. Gómez C, Horna DH, Olano C, Palomino-Schätzlein M, Pineda-Lucena A, Carbajo RJ, Braña AF, Méndez C, Salas JA (2011) Amino acid precursor supply in the biosynthesis of the RNA polymerase inhibitor streptolydigin by *Streptomyces lydicus*. J Bacteriol 193:4214–4223

15. Horna DH, Gómez C, Olano C, Palomino-Schätzlein M, Pineda-Lucena A, Carbajo RJ, Braña AF, Méndez C, Salas JA (2011) Biosynthesis of the RNA polymerase inhibitor streptolydigin in *Streptomyces lydicus*: tailoring modification of 3-methyl-aspartate. J Bacteriol 193(10):2647–2651

16. Jonsbu E, Christensen B, Nielsen J (2001) Changes of in vivo fluxes through central metabolic pathways during the production of nystatin by *Streptomyces noursei* in batch culture. Appl Microbiol Biotechnol 56(1–2):93–100

17. Kim YJ, Song JY, Moon MH, Smith CP, Hong SK, Chang YK (2007) pH shock induces overexpression of regulatory and biosynthetic genes for actinorhodin production in *Streptomyces coelicolor* A3(2). Appl Microbiol Biotechnol 76(5):1119–1130

18. Kirk S, Avignone-Rossa CA, Bushell ME (2000) Growth limiting substrate affects antibiotic production and associated metabolic fluxes in *Streptomyces clavuligerus*. Biotechnol Lett 22(22):1803–1809

19. Kol S, Merlo ME, Scheltema RA, de Vries M, Vonk RJ, Kikkert NA, Dijkhuizen L, Breitling R, Takano E (2010) Metabolomic characterization of the salt stress response in *Streptomyces coelicolor*. Appl Environ Microbiol 76(8):2574–2581

20. Li XB, Qiao B, Yuan YJ (2006) Differential analysis of secondary metabolites by LC–MS following strain improvement of *Streptomyces lydicus* AS 4.2501. Biotechnol Appl Biochem 45(Pt 3):107–118

21. Li LZ, Zheng H, Yuan YJ (2007) Effects of propionate on streptolydigin production and carbon flux distribution in *Streptomyces Lydicus* AS 4.2501. Chin J Chem Eng 15(2):143–149

22. Li M, Kim TJ, Kwon HJ, Suh JW (2008) Effects of extracellular ATP on the physiology of *Streptomyces coelicolor* A3(2). FEMS Microbiol Lett 286:24–31

23. Lounès A, Lebrihi A, Benslimane C, Lefebvre G, Germain P (1995) Glycerol effect on spiramycin production and valine catabolism in *Streptomyces ambofaciens*. Curr Microbiol 31(5):304–311

24. May WS Jr, Formica JV (1978) Glutamate-induced uptake of proline by *Streptomyces antibioticus*. J Bacteriol 134(2):546–554

25. Olano C, Gómez C, Pérez M, Palomino M, Pineda-Lucena A, Carbajo RJ, Braña AF, Méndez C, Salas JA (2009) Deciphering biosynthesis of the RNA polymerase inhibitor streptolydigin and generation of glycosylated derivatives. Chem Biol 16:1031–1044

26. Pearce CJ, Rinehart KL Jr (1983) The use of doubly labeled 13Cacetate in the study of streptolydigin biosynthesis. J Antibiot 36:1536–1538

27. Pronin SV, Kozmin SA (2010) Synthesis of streptolydigin, a potent bacterial RNA polymerase inhibitor. J Am Chem Soc 132(41):14394–14396

28. Rodriguez E, Navone L, Casati P, Gramajo H (2012) Impact of malic enzymes on antibiotic and triacylglycerol production in *Streptomyces coelicolor*. Appl Environ Microbiol 78(13):4571–4579

29. Smith DD, Wood NJ, Hodgson DA (1995) Interaction between primary and secondary metabolism in *Streptomyces coelicolor* A3(2): role of pyrroline-5-carboxylate dehydrogenase. Microbiology 141(Pt 7):1739–1744

30. Temiakov D, Zenkin N, Vassylyeva MN, Perederina A, Tahirov TH, Kashkina E, Savkina M, Zorov S, Nikiforov V, Igarashi N, Matsugaki N, Wakatsuki S, Severinov K, Vassylyev DG (2005) Structural basis of transcription inhibition by antibiotic streptolydigin. Mol Cell 19(5):655–666

31. Vassylyev DG, Vassylyeva MN, Zhang J, Palangat M, Artsimovitch I, Landick R (2007) Structural basis for substrate loading in bacterial RNA polymerase. Nature 448(7150):163–168

32. Voelker F, Altaba S (2001) Nitrogen source governs the patterns of growth and pristinamycin production in 'Streptomyces pristinaespiralis'. Microbiology 147(Pt 9):2447–2459

33. Zhao GR, Luo T, Zhou YJ, Jiang X, Qiao B, Yu FM, Yuan YJ (2009) fabC of Streptomyces lydicus involvement in the biosynthesis of streptolydigin. Appl Microbiol Biotechnol 83(2):305–313