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Enhancement of ansamitocin P-3 production in *Actinosynnema pretiosum* by a synergistic effect of glycerol and glucose

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Abstract Ansamitocin P-3 (AP-3), a secondary metabolite produced by *Actinosynnema pretiosum*, is well known for its extraordinary antitumor properties and is broadly utilized in clinical research. Through this work, we found, for the first time, that the combination of glucose and glycerol as a mixed carbon source is an appropriate approach for enhancing the production of AP-3 by *A. pretiosum*. The amount yielded was about threefold that obtained with glucose as the sole carbon source. In order to better understand the mechanisms that channel glycerol metabolism towards AP-3 production, the activities of some key enzymes such as glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, phosphoglucomutase (PGM), and fructose 1,6-bisphosphatase were assessed. The results showed that glycerol affects the production of AP-3 by increasing PGM activity. Furthermore, qRT-PCR analysis revealed that transcriptional levels of structural genes *asm14* and *asm24*, and primary genes *amir5189* and *amir6327* were up-regulated in medium containing glycerol.

Keywords *Actinosynnema pretiosum* · Ansamitocin P-3 · Glycerol · Metabolic regulation · Transcriptional level

Introduction

Maytansinoids are extraordinarily strong antitumor agents generally isolated from living organisms such as plants and mosses [4]. An actinomycete, *Actinosynnema pretiosum*, similarly displays the capacity to secrete large amounts of various maytansinoids known as ansamitocins. Ansamitocins are a series of complex aromatic compounds including AP-0, AP-2, AP-3, AP-3′, and AP-4, among which ansamitocin P-3 (AP-3) is confirmed to be the active ingredient [17]. AP-3 has been paid a lot of attention in recent years because of its industrial importance. Biosynthesis of ansamitocin in *A. pretiosum* involves a sequence of reactions that require the starter unit 3-amino-5-hydroxybenzoic acid (AHBA) [10] from which is formed proansamitocin through the action of a polyketide synthase. The resulting proansamitocin is further converted through six tailoring steps into the bioactive end product, ansamitocin P-3 (AP-3) [24, 25, 28] (Fig. 1). Many carbon sources have been screened with the objective of setting up a culture medium that would improve the production of AP-3 in large-scale industrial fermentation.

Glycerol, which forms as the co-product of biodiesel at a weight ratio of about 10 % [5], is considered as a cheap, abundant, highly reduced carbon source that is used widely in microbial fermentation [6]. Numerous reports have shown that glycerol can be considered as a promising compound that could significantly increase the production yield of various chemicals. For instance, the use of glycerol supported better growth of *Streptomyces hygroscopicus* $D_{1.5}$ and was beneficial to the production of antibiotics. It can also increase the titer of spiramycin produced by *Streptomyces spiramyceticus* [1, 11].

Feeding experiments showed that glycerol-derived 3-carbon starter units, 2-carbon extender units, or 3-carbon units with branches in their hydrocarbon chains are preferentially incorporated during the biosynthesis of several polyketide secondary metabolites [26]. Classical extender units in polyketide biosynthesis are malonyl-CoA and

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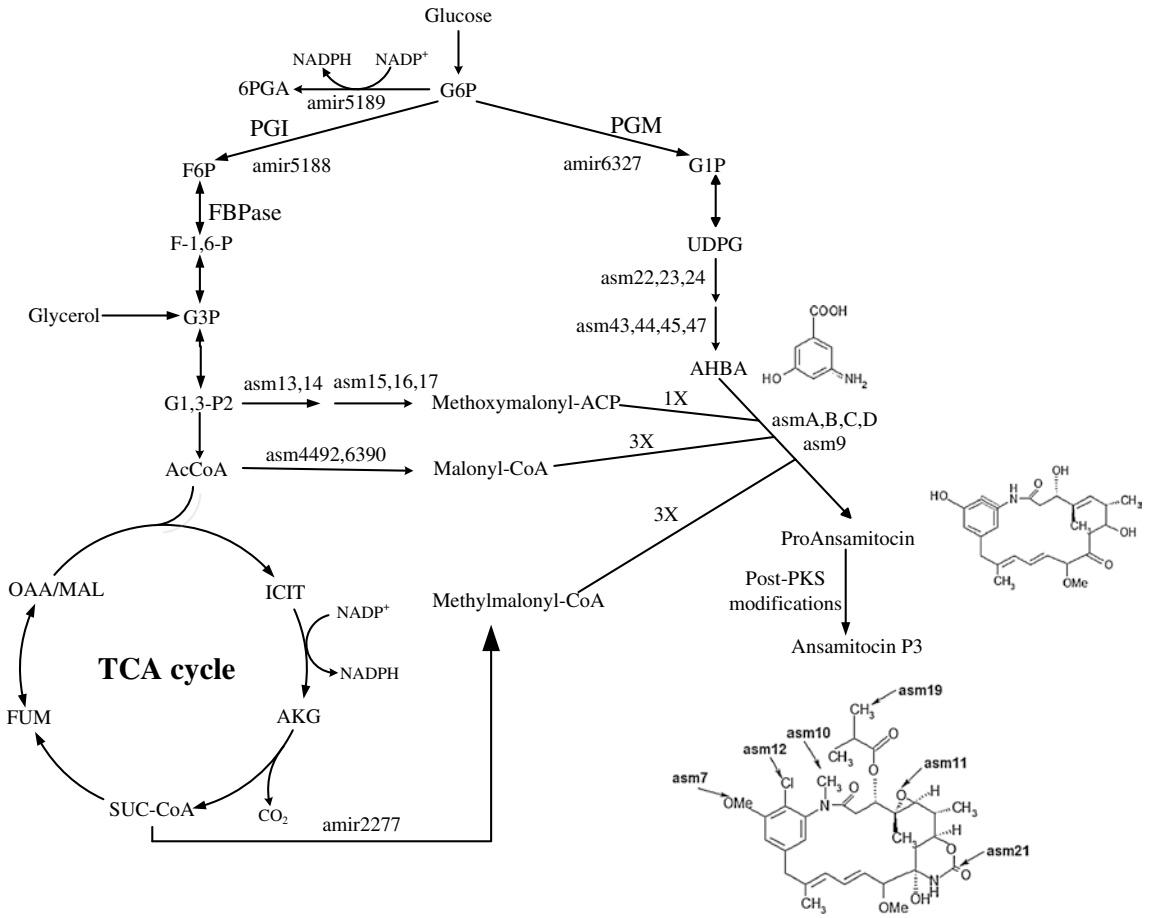

Fig. 1 Simplified pathways for ansamitocin P-3 (AP-3) biosynthesis

methylmalonyl-CoA [21]. Recently, several examples of the utilization of glycerol-derived extender units, which result in the incorporation of β-methoxy or β-hydroxy 2-carbon units into a polyketide chain, have been reported. Indeed, glycerol is a pivotal precursor at the center of several primary metabolic pathways including the pentose phosphate pathway and the shikimate pathway [18]. Despite the aforementioned properties, no study has been conducted to elucidate the effect of glycerol on the production of the high value-added metabolite AP-3.

The present survey aimed to investigate the effect of glycerol on AP-3 production by *A. pretiosum* subsp. *auranticum* ATCC 31565. Since this strain is unable to grow when glycerol is used as the sole carbon source, co-fermentation of glucose and glycerol was performed in different glucose/glycerol weight ratios and in different feeding approaches in order to find the best medium composition that leads to the improvement of AP-3 production. Furthermore, activities of key enzymes and transcriptional levels of essential genes implicated in AP-3 biosynthesis were determined in order to analyze the regulation mechanism of AP-3 metabolism in glycerol feeding conditions and provide understandable tools for its production on an industrial scale.

Materials and methods

Strains and cultivation

A. pretiosum ssp. *auranticum* ATCC 31565 was generously provided by the Laboratory of Molecular Microbiology at Shanghai Jiao Tong University, China. The strain was seeded by inoculating 1 ml of the thawed cell suspension in a 250 ml Erlenmeyer flask containing 50 ml of culture medium (glycerol 10 g l^{-1}, yeast extract 5 g l^{-1}, peptone 10 g l^{-1}, glucose 5 g l^{-1}). After 48 h of incubation (28 °C, 220 rpm), 500 μl of the culture was subsequently transferred to 50 ml of fresh culture medium and precultured in the above conditions for a further 24 h. Following this, 500 μl of the preculture was inoculated into 50 ml of M6 medium (NH_4Cl 2 g l^{-1}, KH_2PO_4 0.65 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g l^{-1}, $FeSO_4{\cdot}7H_2O$ 0.01 g l^{-1}, $CaCO_3$ 0.5 g l^{-1}, glucose 10 g l^{-1} (or glucose 5 g l^{-1} + glycerol 5 g l^{-1}), pH 6.8 and

incubated at 28 °C under agitation conditions (220 rpm) for 8 days. Samples were taken every 24 h until the end of the fermentation.

Analytical methods

After centrifuging 3 ml of samples, the supernatant was used for the analysis of extracellular compounds and cell pellets were used for the determination of dry cell weight (DCW). The pellets were washed and recentrifuged twice with 0.1 M HCl in order to remove $CaCO_3$, and dried at 105 °C for approximately 24 h until the weight remained constant. For the analysis of extracellular metabolites, the supernatant was filtered through a 0.45 μm syringe filter and HPLC assay was conducted. The HPLC system was equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) operating at 60 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. The residual glucose and glycerol were quantified with a differential refractometer.

For analysis of antibiotic production, 3 ml of the culture samples was centrifuged at 10,000*g* for 5 min and AP-3 was extracted from 2 ml of the supernatant with 2 ml of ethyl acetate (three times). Subsequent to the removal of the ethyl acetate solvent with the help of rotary evaporation at 40 °C, the purified residue was dissolved in 1 ml methanol and the extracted AP-3 was analyzed using a Shimadzu LC-20AD HPLC system (Shimadzu Corporation, Tokyo, Japan) with a C18 column using 85 % methanol and 15 % water as mobile phase at the flow rate of 0.6 ml min^{-1}. The column temperature was 30 °C. The UV detector was set at 254 nm. Pure AP-3 (purity >98 %) was applied as a standard for the establishment of the calibration curve. The cultivations were all performed in triplicate.

Enzyme assays

The initial reaction mixture for phosphoglucomutase (PGM) (100 μl) consisted of 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM glucose 1,6-bisphosphate, 5 mM glucose-1-phosphate (G1P), and cell-free extract. The amount of glucose-6-phosphate (G6P) produced was measured by addition of 400 μl of water containing 0.5 mM $NADP^+$ and 2 U of glucose-6-phosphate dehydrogenase (G6PDH). After incubation at 25 °C for 3 min, the amount of NADPH was measured at 340 nm [29].

The glucose-6-phosphate isomerase (PGI) activity assay solution contained 0.1 M Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 0.5 mM $NADP^+$, 1 U G6PDH, and 2 mM fructose-6-phosphate (F6P) [23]. Glucose 6-phosphate dehydrogenase activity was obtained by determining the rate of NADPH formation at 340 nm [13].

The fructose 1,6-bisphosphatase (FBPase) activity was determined with a solution of 50 mM imidazole–HCl (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.25 mM $NADP^+$, 0.1 mM fructose-1,6-diphosphate (FDP), 0.5 U PGI, 0.5 U G6PDH, and the concentration of NADPH was measured at 340 nm [9].

Transcriptional analysis by quantitative real-time RT-PCR

Microbial cells were collected from the fermentation medium and total RNA was extracted using Trizol reagent (Sigma-Aldrich Co., St Louis, MO) according to the instruction of the manufacturer, and treated with DNase I (Thermo Fisher Scientific Inc., Waltham, MA) to remove residual genomic DNA. Reverse transcription was achieved with a ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan).

Transcriptional levels were determined by quantitative real-time RT-PCR (qRT-PCR) using an SYBR Green qPCR Master MIX (2×) Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instruction. Reaction conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at optimal temperature, and 30 s at 72 °C. For each gene, the expression level of the control sample was defined as 1.0 and the results were shown as the fold of mRNA level over the control sample. The $2^{-\Delta\Delta C_t}$ method was applied to analyze the data of qRT-PCR [16]. Primers for qRT-PCR assay in this study are listed in Table 1.

Results and discussion

Effect of different glucose/glycerol ratios on the metabolism of *A. pretiosum* and AP-3 production

In our preliminary experiments, different glucose/glycerol ratios were established by varying glycerol concentrations from 1 to 15 g l^{-1} and fixing glucose concentration at 10 g l^{-1}. The effect of glycerol concentrations on AP-3 production by *A. pretiosum* in shake-flask fermentation was examined (Table 2). The results showed that under cultivation conditions with 5–15 g l^{-1} glycerol concentration, the amount of AP-3 produced was obviously increased in each defined culture. AP-3 productions were similar when glycerol addition varied from 5 to 15 g l^{-1} (highest AP-3 concentration of about 13.9 mg l^{-1} was obtained in the case of 15 g l^{-1} glycerol, slightly greater than other cases). An about 40 % decrease in AP-3 production was, however, observed when glycerol addition dropped to 3 g l^{-1}. The AP-3 production was also minute in the original M6 medium with 10 g l^{-1} glucose as the sole carbon source, and supplementation of a small amount of glycerol (e.g.,

Table 1 Primers used for quantity qRT-PCR

Gene	Primer sequences
amir5188	5′ GCTGCCGAAGGACGCCAAGA 3′
	5′ CGTTCTGGTGACCGCCCTTGTG 3′
amir5189	5′ AGACCTACGCCGCCATCACC 3′
	5′ TGCCCGTAGCCGAAGTCCA 3′
amir6327	5′ ACGGCAACTTCCCGAACCACG 3′
	5′ AGTGCTCGCCGCCGAAGATG 3′
asm4492	5′ CCAAGCGGCTGCTCAACGA 3′
	5′ GGTCGTTGAGCGAGGTGTTGC 3′
asm6390	5′ ACACCAAGTCGGGCAACGC 3′
	5′ GGCTGCACCTCCAGGAACTCA 3′
amir2277	5′ TTCACCTACCTCGTGGACCTGC 3′
	5′ GCCCTCCAGGCAGTTCATCC 3′
asm14	5′ CGGCCTGTCGTCGCTGTT 3′
	5′ GCACCGTGCGGAAGTTGT 3′
asm19	5′ GCCCCTACTTCCGACGCC 3′
	5′ CGCCAGGAGCAGCAGCAC 3′
asm24	5′ CAGCAGCAGACCGAC 3′
	5′ GGGACCTGGAACATG 3′
asm29	5′ GCTGCTCACCACGGGC 3′
	5′ CAGCATCGTGCGGACG 3′
asm43	5′ GCCCGACCTGACCCCCG 3′
	5′ CGCCCAGCAGCACCCG 3′
16s RNA	5′ CAGAAGAAGCACCGGCTAAC 3′
	5′ TTAAGCCCCAAGTTTTCACG 3′

1 g l^{-1}) did not increase the antibiotic synthesis significantly. The aforementioned results suggest that the combination of glucose with an appropriate concentration of glycerol could effectively enhance the production of AP-3.

The kinetic profiles of glucose and glycerol consumption are depicted in Fig. 2. As shown in Fig. 2a, glucose was depleted after 72 h cultivation in all media and the uptake rate was the fastest from 48 to 72 h. However, glycerol was completely consumed within 168 h when the additive amount was 1, 3, and 5 g l^{-1} (Fig. 2b). On the contrary, at 7, 10, and 15 g l^{-1} glycerol concentration, residual glycerol was observed in the culture medium, showing that glycerol was present in excess at these ratios. Moreover, these results revealed that glycerol plays an important role in the biosynthesis of AP-3 by *A. pretiosum* ssp. *auranticum* ATCC 31565. With the help of glycerol, antibiotic production was increased by about sixfold when medium cultures contain extra glycerol at 5 g l^{-1}.

Cultivations on equimolar amounts of carbon source

Another experiment with reduced amounts of glucose was conducted to clarify that the improvement of AP-3 production was not related to the extra amount of carbon source. The weight ratio of glucose/glycerol in cultures was fixed at 5:5 (g l^{-1}/g l^{-1}) to ensure that the molar amount of carbon was equivalent to 10 g l^{-1} glucose. Fermentation profiles of AP-3 production, cell growth, pH variation, and

Table 2 Effect of the addition of glycerol on the production of AP-3

Conditions	AP-3 production (mg l^{-1})	Conditions	AP-3 production (mg l^{-1})
10 g l^{-1} glucose	2.20 ± 0.22		
10:1 glucose/glycerol	3.15 ± 0.53	10:7 glucose/glycerol	12.03 ± 0.87
10:3 glucose/glycerol	8.53 ± 0.62	10:10 glucose/glycerol	12.35 ± 1.11
10:5 glucose/glycerol	13.06 ± 0.89	10:15 glucose/glycerol	13.86 ± 1.22

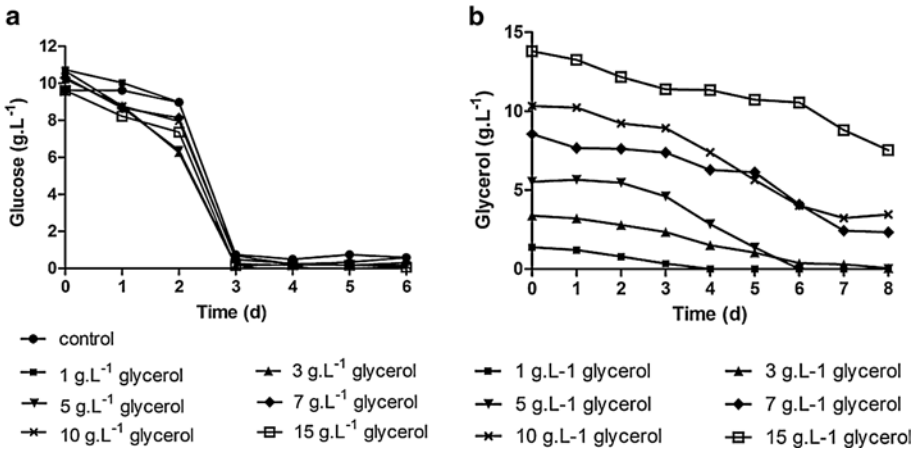

Fig. 2 Effects of the addition of different concentrations of glycerol on the consumption of glucose(**a**) and glycerol (**b**)

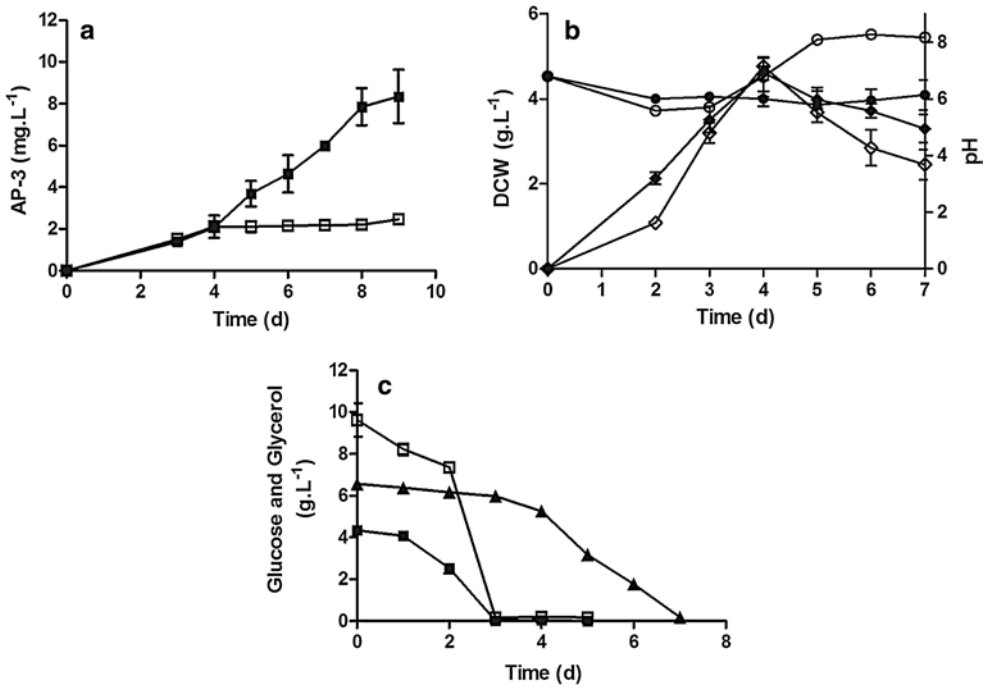

Fig. 3 Fermentation profiles of 10 g l^{-1} glucose and 5:5 glucose/glycerol ratio. Cells were grown in shake-flask cultures containing 10 g l^{-1} glucose (*open symbols*), and 5 g l^{-1} glucose adding 5 g l^{-1} glycerol (*closed symbols*) as carbon source, respectively. **a** AP-3 production (*squares*), **b** dry cell weight (*diamonds*), pH (*circles*), and **c** consumption of glucose (g l^{-1}) (*squares*) and glycerol (*triangles*)

glucose and glycerol consumption in glucose and glucose/glycerol cultures are shown in Fig. 3.

In the 5:5 glucose/glycerol ratio condition, experimental results of the shake-flask culture revealed that at the end of the cultivation, AP-3 production was 8.4 mg l^{-1}, about 3.4-fold higher than the control (2.5 mg l^{-1}) (Fig. 3a), whereas with a weight glucose/glycerol ratio of 10:5, this value was 13.0 mg l^{-1} (Table 2). A similar situation was also found in another report on the production of apramycin when half of the glucose was replaced by glycerol [2], which corroborates the results obtained in this study. These results demonstrated that even when carbon molar amounts were the same, the tendency of glycerol to be employed for AP-3 production was still better than that of glucose.

Time profiles of DCW and medium pH are shown in Fig. 3b, indicating that the addition of glycerol resulted in up to a 50 % increase in cell growth before day 4. After maximum cell concentrations were achieved at day 4, the DCW values dropped quickly for both 10:0 and 5:5 glucose/glycerol cases, which might be attributed to biomass autolysis caused by the alkaline pH environment during the later period of fermentation. As indicated in this figure, the medium pH increased from day 4 and reached a maximum of 8.28 on day 6 with the medium without glycerol, whereas the other series was maintained in the vicinity of 6.0 during the whole fermentation process. The exhaustion of glucose in the medium with glucose as the sole carbon source was followed by the rapid increase of the medium pH value. This phenomenon was relieved with the presence of glycerol in the medium. Similar pH variation was also observed in clavulanic acid production with a glycerol feed [12]. In our case, the pH decline at the beginning of the fermentation might be due to the production of certain organic acids, whereas the pH increase towards the end of fermentation was considered to be related to cell autolysis, the increase of pH was always accompanied by biomass decline. As shown in Fig. 3b, the pH did not increase substantially at the end of fermentation if glycerol was added, indicating that feeding glycerol might prolong the stage of active cell metabolism.

In medium containing glucose plus a more slowly utilized carbon source, glucose is usually used first during the non-antibiotic production phase. After glucose depletion, the second carbon source is then used for antibiotic biosynthesis [8]. This phenomenon was also found in our study. As *A. pretiosum* is unable to grow when glycerol is used as a sole carbon source, glucose can be used to stimulate the biomass accumulation. In medium containing glucose and glycerol, glucose was apparently used initially and rapidly to produce biomass but few or no secondary metabolites were synthesized. After the depletion of glucose, glycerol was used as the "second-best" carbon source for secondary metabolite biosynthesis. In these conditions, glucose was exhausted at day 3 whilst almost no glycerol was utilized

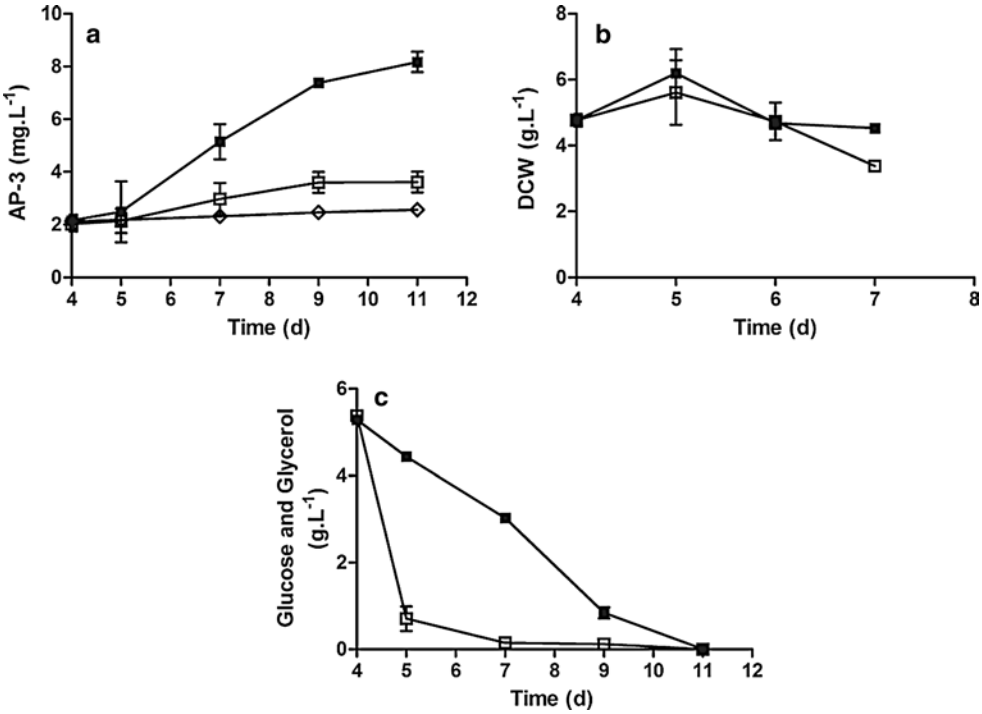

Fig. 4 **a** Effects of adding glucose and glycerol after depletion of glucose on AP-3 production. **b** Dry cell weight. **c** Consumption of glucose and glycerol. 10 g l^{-1} glucose (*open diamonds*); feeding 5 g l^{-1} glucose (*open squares*) and 5 g l^{-1} glycerol (*closed squares*) after 10 g l^{-1} glucose was depleted (day 4)

during this period. The consumption of glycerol began after day 3 and continued until day 7 without glycerol exhaustion (Fig. 3c).

Effects of feeding glucose and glycerol on AP-3 production and consumption of carbon sources

To investigate respective contributions of glucose and glycerol to AP-3 biosynthesis, these two carbon sources were separately added into the culture medium after the exhaustion of the initial 10 g l^{-1} glucose (day 4). AP-3 production was detected from day 4 to day 11. As shown in Fig. 4a, feeding glycerol at the fourth day of fermentation led to an AP-3 yield of 7.4 mg l^{-1} whereas the use of glucose instead of glycerol yielded 3.6 mg l^{-1} AP-3. AP-3 production in these two conditions was about 3- and 1.5-fold higher, respectively, versus the control. On the other hand, the additional 5 g l^{-1} glucose was almost used up during 1 day. The consumption rate of glycerol was much slower than that of glucose. Glycerol was consumed gradually over the next 5 days after its addition into the medium (Fig. 4c). Obviously, the addition of extra carbon sources during the fermentation process can provide more precursor sources for AP-3 biosynthesis and then further increase AP-3 production. In addition, these extra carbon sources helped to raise the cell growth to some extent and maintain the DCW at a higher level (Fig. 4b). Our results showed that glycerol is better than glucose in terms of channeling the carbon flux to AP-3 biosynthesis.

Enzymatic activities in different carbon source conditions

In our preliminary experiment, exogenous 5 mm AHBA was added into M6 medium (10 g l^{-1} glucose as carbon source), and the concentration of AP-3 increased to 6.2 mg/l, which was twofold higher than that of the culture without AHBA addition. The precursor AHBA was therefore one of the limiting factors for AP-3 biosynthesis. Four key enzymes, G6PDH, PGI, PGM, and FBPase, were assessed with the purpose of analyzing the effects of glucose and glycerol on the flux distribution of the pentose phosphate pathway (PPP), glycolytic pathway (EMP), G1P biosynthesis, and gluconeogenic pathways.

G6P is the first branch node in central carbon metabolism, and PGI is the first enzyme in the glycolytic pathway. As shown in Fig. 5a, significant enhancement of this enzyme's activity was observed from day 5 to day 7 in glucose-supplemented cultures with a maximum value of 0.51 U/mg. This can be explained by the fact that *A. pretiosum* ssp. *auranticum* ATCC 31565 cells synthesized considerable amounts of PGI in order to interconvert G6P into F6P and enter into the glycolytic pathway. The glycolytic pathway was far more active in glucose-supplemented cultures than glycerol-supplemented cultures. However, as glycerol is needed for the synthesis of G6P through the gluconeogenic pathway, it was consumed gradually throughout the remaining incubation time. PGI activity increased gradually and reached a maximum value of 0.23 U/mg on day 7. The intracellular G6P content in glycerol-supplemented cultures might be less than that in glucose-supplemented

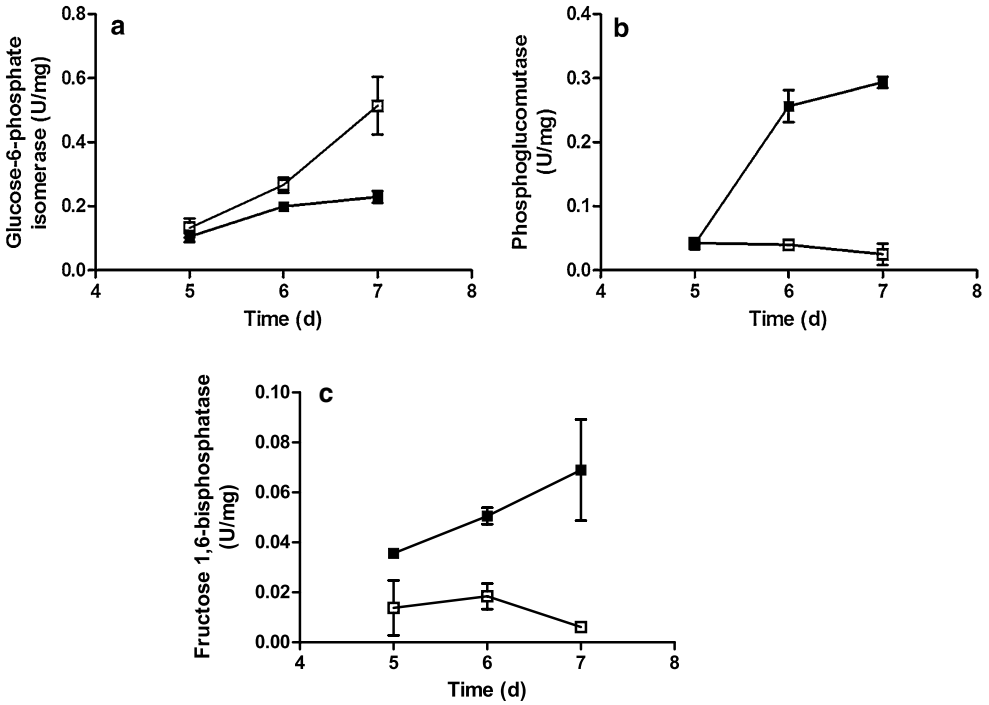

Fig. 5 Changes of enzymatic activities with different carbon source on day 5, day 6, and day 7. **a** Glucose-6-phosphate isomerase. **b** Phosphoglucomutase. **c** Fructose 1,6-bisphosphatase. Feeding 5 g l^{-1} glucose (*open squares*) and 5 g l^{-1} glycerol (*closed squares*) after 10 g l^{-1} glucose was depleted (day 4)

cultures, PGI activities showed a similar trend under these two conditions.

PGM catalyzes the conversion of G6P into G1P and subsequently directs the flux towards UDP-glucose, an important precursor of AHBA. The maximum PGM activity levels were 0.04 U/mg in glucose-supplemented cultures (day 5) and 0.29 U/mg in glycerol-supplemented cultures (day 7). When feeding glucose on day 4, the activity of PGM declined gradually and reached the minimum value on day 7. On the contrary, when glucose was replaced by glycerol, this activity increased substantially from 0.04 U/mg on day 5 to 0.26 and 0.29 U/mg on day 6 and day 7, respectively (Fig. 5b). These changes in PGM activity were in accordance with AP-3 production. During day 4 to day 5, the increments of AP-3 were almost the same, and PGM activities were also similar to each other. During the next several days, in glycerol-supplemented cultures, AP-3 was produced in much greater amounts than in glucose cultures, and this was consistent with the high activity of PGM in these cultures. In addition, as the additional 5 g l^{-1} glucose was almost used up during 1 day, PGM activity levels were not as high as in glycerol-supplemented cultures, suggesting that the addition of glycerol is vital for the activation and the maintenance of the enzymatic activity of PGM in the strain used. The improvement of AP-3 production might be due to the enhancement of the flux through this pathway in concert with the release of the limitation of AHBA supply.

G6PDH is the first enzyme in PPP. The activity level of this enzyme was too low to be detected; therefore qRT-PCR data will be used to discuss and explain its activity in the sections below.

FBPase, which catalyzes the biosynthesis of F6P from FDP, is a rate-limiting enzyme of the gluconeogenic pathway. The activity of FBPase increased with time and reached a maximum level of 0.07 U/mg on day 7 in glycerol-fed cultures. In glucose-supplemented cultures, FBPase activity was greatly reduced compared to glycerol-supplemented cultures. The carbon flow through the gluconeogenic pathway was highly increased when glycerol was used as a carbon source (Fig. 5c). Glycerol was used to generate more G6P through the gluconeogenic pathway, whereas glucose was directly converted into G6P and then mainly processed through the glycolytic pathway. The backflow of carbon sources to G6P through the gluconeogenic pathway probably retuned the flow direction originating from G6P. G1P generation was then stimulated to provide more AHBA precursors and increase AP-3 production in this study.

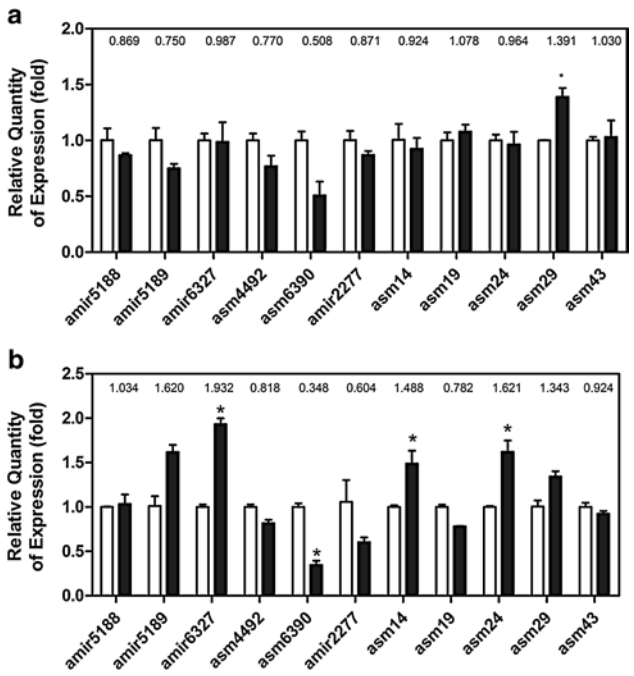

Fig. 6 Effects of glucose and glycerol on gene expression. qRT-PCR was performed after additional 5 g l^{-1} glucose (*open bars*) and 5 g l^{-1} glycerol (*filled bars*) were added into the cultures on the fourth day of the incubation. The figure shows different gene transcriptional levels in response to glucose and glycerol on day 5 (**a**) and day 6 (**b**). The relative quantity of gene expression of the *open column* is fixed to 1.0, the *number* (fold) at the *top* of the figure represents the relative quantity of gene expression of the *filled column*. The values are the averages of two series of duplicate experiments, $^*P < 0.05$. *amir5188* glucose-6-phosphate isomerase, *amir5189* glucose-6-phosphate dehydrogenase, *amir6327* phosphoglucomutase, *asm4492* subunit of acetyl-CoA carboxylase, *asm6390* carboxyl transferase, *amir2277* methylmalonyl-CoA mutase, *asm14* ACP (D-alanyl carrier protein), *asm19* 3-*O*-acyltransferase, *asm24* AHBA synthase, *asm29* transcriptional regulator, *asm43* AHBA synthase

Effects of feeding glucose and glycerol on gene expression

Transcriptional response of G6P metabolic node

The expression of three genes, *amir5188*, *amir5189*, and *amir6327*, encoding respectively PGI, G6PDH, and PGM and therefore representative of the flow distribution from the G6P node was investigated. As the extra glucose was almost depleted in 1 day, we chose day 5, when glucose was used up, and day 6, when glucose was totally metabolized in metabolic pathways, to compare differences in gene expression levels under these two carbon source conditions. As shown in Fig. 6, *amir5188* and *amir5189* were down-regulated in glycerol-supplemented cultures on day 5 whereas *amir6327* showed no obvious difference between the two conditions, suggesting that the flux through the EMP and PPP was stronger when glucose was used. On the contrary, on day 6, *amir5189* and *amir6327* showed a 1.6- and 1.9-fold increase in glycerol-supplemented versus glucose-supplemented cultures, indicating that PPP and G1P generation declined after glucose depletion. These results are in accordance with enzyme activities data, reflecting comprehensively that glucose is mainly processed via the EMP whereas glycerol is partly used through gluconeogenesis and further to generate a certain amount of G1P. The flux through the EMP, PPP, and G1P generation pathway can be maintained for a long time during the glycerol consumption period. In cultures provided with glycerol, the AHBA supply may not be a limiting factor any more.

Transcriptional response of PKS precursor supply pathways

The biosynthesis of AP-3 in *A. pretiosum* involves the assembly of a polyketide by chain extension of the starter unit AHBA by three molecules of malonyl-CoA and 2-methylmalonyl-CoA, respectively, and one molecule of 2-methoxymalonyl-acyl carrier protein (ACP) on a type I modular polyketide synthase (asm PKS). The *asm4492* gene is predicted to encode a subunit of acetyl-CoA carboxylase. Another selected gene, *asm6390*, belongs to a family of carboxyl transferase genes. Both the *asm4492* and *asm6390* genes are known to be responsible for the transformation of acetyl-CoA to malonyl-CoA. Downregulation of these two genes in glycerol-supplemented cultures suggested that the supply of malonyl-CoA might be enough for AP-3 biosynthesis. A similar phenomenon was also found for the *amir2277* gene, which encodes the enzyme that catalyzes the generation of methylmalonyl-CoA.

In the ansamitocin cluster (asm), genes *asm13–17* are involved in the synthesis of the unusual "glycolate" unit (methoxymalonyl-ACP) and its delivery to the PKS [3]. The *asm14* gene is a structural gene that encodes for acyl carrier protein in the "glycolate" unit [24]. In this system, the carbon pairs of the "glycolate" unit are derived from the two carbons of glycerol [27]. In our study, the transcriptional level of *asm14* was higher in glycerol medium when glucose was used up, suggesting that glycerol might provide more methoxymalonyl-ACP units in the AP-3 biosynthesis process.

Transcriptional response of other AP-3 biosynthetic genes

It was reported that under conditions of excess ammonium, the AHBA synthase-encoding genes, *asm24* and *asm43*, would be down-regulated to further repress AP-3 yield [14], but no remarkable expression diversity of *asm43* was observed in our study. On the other hand, the relative amount of *asm24* expression in glycerol feeding medium was 1.62 times higher than

Table 3 Effect of addition of glycerol on the dry cell weight, AP-3 production, AP-3 yield, and pH variation

Conditions	Dry cell weight (max)[a] (g l^{-1})	AP-3 production[b] (mg l^{-1})	Overall AP-3 yield[c] (mg g^{-1})	Terminal pH
10 g l^{-1} glucose	3.4 ± 0.42	2.56 ± 0.12	1.40 ± 0.23	8.17 ± 0.03
5:5 glucose/glycerol	3.72 ± 0.16	8.35 ± 1.27	2.53 ± 1.33	6.14 ± 0.53
5 g l^{-1} glucose supplemented[d]	5.23 ± 0.39	3.61 ± 0.39	0.95 ± 0.05	6.81 ± 0.49
5 g l^{-1} glycerol supplemented[e]	5.07 ± 0.25	8.17 ± 0.39	2.21 ± 0.14	6.15 ± 0.06

[a] Maximum dry cell weight during the cultivation

[b] AP-3 production at the end of the cultivation

[c] The ratio of AP-3 production and dry cell weight at the end of the cultivation

[d] Feeding 5 g l^{-1} glucose into the medium after the initial glucose was depleted

[e] Feeding 5 g l^{-1} glycerol into the medium after the initial glucose was depleted

glucose-supplemented medium on day 6, suggesting that *asm24* might play a dominant role in AHBA generation in glycerol-supplemented cultures. The *asm29* gene is a regulatory gene in the process of AP-3 biosynthesis and its expression was reported to be up-regulated in a high AP-3 producer [20]. Furthermore, it was reported that the regulatory gene *asm29* had no significant variation with or without ammonium or isobutanol supplementation in the culture medium [14, 15]. As shown in Fig. 6, the expression of *asm29* was 1.39 and 1.34 times higher when glycerol was used as the main carbon source for AP-3 synthesis on day 5 and day 6. This indicated that *asm29* was a positive regulator in our growth conditions.

As reported previously, *asm19* encodes an acyltransferase which delivers the acyl group from the corresponding acyl-CoA to 3-O [19]. As shown in Fig. 6, there was no marked change for *asm19* expression between the two conditions, suggesting that adding glycerol might not contribute to this post-modification step and that *asm19* might not respond to glycerol stimulation.

Conclusion

The carbon sources needed for maximal antibiotic production seem to be different among bacterial strains. The biosynthetic pathways leading to the production of secondary metabolites such as antibiotics are often connected to and influenced by pathways of primary metabolism [7]. A primary pathway frequently provides the precursors for the antibiotic molecule.

The current study showed that glucose/glycerol co-fermentation can serve as an efficient strategy for ansamitocin production by *A. pretiosum* ssp. *auranticum* ATCC 31565. Glycerol addition stimulated the biosynthesis of AP-3 but had little effect on cell growth compared with glucose in equimolar carbon concentration. Some fermentation parameters such as the maximum DCW, AP-3 production, overall AP-3 yield, and pH value are summarized in Table 3. When the weight ratio of glucose/glycerol was 5:5, the production and yield of AP-3 was 8.4 mg l^{-1}, and 2.5 mg g^{-1}, respectively, which was 3.3- and 1.8-fold higher than the amount obtained in the medium containing only glucose as the carbon source. Adding 5 g l^{-1} glycerol in the middle of the fermentation process could similarly increase AP-3 production to 8.2 mg l^{-1}, 2.3-fold higher than adding 5 g l^{-1} glucose. Structural gene *asm14* was up-regulated to a certain extent in glycerol medium, suggesting that glycerol could supply precursor methoxymalonyl-ACP more effectively. As the relative expression of gene *asm24* was higher in glycerol-supplemented cultures, we may draw the conclusion that the ability of glycerol to enhance AP-3 production might be due to the increase of AHBA supply. Elevated expression levels of *amir6327* and high activity of phosphoglucomutase in glycerol-supplemented cultures were observed, indicating the enhancement of the flux from G6P to G1P which was shown to be extremely small based on our ^{13}C labeling experiments (unpublished data). Increasing the flux of this pathway might be one of the key factors for AP-3 production. The *amir6327* gene encodes phosphoglucomutase, which provides the substrate for ADP-glucose pyrophosphorylase, the first enzyme dedicated to glycogen synthesis. Ryu et.al [22] found that deletion of PGM encoded by SCO7443 in *Streptomyces coelicolor* resulted in severe reduction in actinorhodin production. So glycogen may provide carbon for polyketide biosynthesis. The results suggest that it would be worth exploring the consequences of *pgm* overexpression. The strategy has important significance in economic industrial fermentation of this valuable antitumor compound.

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