

RESEARCH

Open Access



Improved methanol-derived lovastatin production through enhancement of the biosynthetic pathway and intracellular lovastatin efflux in methylotrophic yeast

Yiqi Liu¹, Chenxiao Bai¹, Qin Xu¹, Jiahui Yu¹, Xiangshan Zhou¹, Yuanxing Zhang^{1,2} and Menghao Cai^{1*}

Abstract

Background: Methanol has attracted interest as a substrate for improvement of product titers and yields of bio-processes, because methanol has a more reduced state than sugars do and can be renewably synthesized from abundant natural gas supplies. Our aim was to engineer methylotrophic *Pichia pastoris* to convert methanol into value-added lovastatin more productively.

Results: A strengthened biosynthetic pathway of lovastatin was constructed through the assembly of three modules with increasing module-specific antibiotic stress in the methylotrophic yeast *P. pastoris*. The resulting strain (*P. p/LV_V#9*) produced 287.5 ± 2.0 mg/L lovastatin in a 5-L bioreactor from methanol. The production was further improved by identification and overexpression of a statin pump protein, TapA, a membrane protein capable of lovastatin efflux out of the cell. A TapA-overexpressing strain, *P. p/LV_V#9-TapA*, produced 419.0 ± 9.5 mg/L lovastatin from methanol: 46% more than *P. p/LV_V#9* did, and 520% more relative to the strain (*P. p/LV_SC*) with single-copy genes.

Conclusions: A methylotrophic yeast strain producing 419.0 ± 9.5 mg/L lovastatin was constructed by optimization of biosynthetic gene dosages and coexpression of a statin pump protein; these results proved that *P. pastoris* is a promising chassis organism for natural-product biosynthesis. A membrane protein, TapA was found to perform the function of exporting intracellular lovastatin and enhanced lovastatin production.

Keywords: Enhanced biosynthetic pathway, Lovastatin, Methanol, *Pichia pastoris*, Statin pump protein

Background

Methanol, one of the C1 compounds, has attracted considerable interest as a promising feedstock for the production of value-added biological products (Bennett et al. 2018). Methanol can be inexpensively manufactured from natural gas (which is abundant in the deep sea of our planet) and can also be renewably obtained through reduction of carbon dioxide (Räuchle et al. 2016). Meanwhile, methanol possesses a higher degree of reduction than traditional sugars do (Whitaker et al. 2015). Although efforts have been focused on engineering

native or synthetic methylotrophs to convert methanol for the production of biochemical metabolites, the effects are still at the rudimentary level owing to the limited genetic tools for native methylotrophs and the weak methanol consumption abilities of the artificial modified methylotrophs (Whitaker et al. 2015; Price et al. 2016). *Pichia pastoris* is a natural methylotrophic yeast that can utilize methanol for growth to high cell densities (Yang and Zhang 2017). It has been researched for years, and a variety of genetic tools and expression vectors has been commercialized (Cregg et al. 2000). Therefore, *P. pastoris* is well suited as a chassis cellular system to convert methanol to various value-added compounds (Schwarzahns et al. 2017).

*Correspondence: cmh022199@ecust.edu.cn

¹ State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
Full list of author information is available at the end of the article

Lovastatin, a natural polyketide drug isolated from *Aspergillus terreus*, is used to treat hypercholesterolemia owing to its (3S)-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase-inhibitory activity (Campbell and Vederas 2010). Industrial lovastatin is mainly produced by fungal fermentation; however, fermentation using native *A. terreus* usually poses some problems such as a long culture period, difficult manipulation, and multiple byproducts (Vogl et al. 2013; Billingsley et al. 2016). With the development of synthetic biology, reconstruction of biosynthetic pathways in chassis organisms has been proved to be a possible solution to these problems (Ro et al. 2006; Galanie et al. 2015). The biosynthetic gene cluster and catalytic role of each functional gene product are well characterized for lovastatin (Kennedy et al. 1999; Xie et al. 2006; Barriuso et al. 2011; Xu et al. 2013). Previously, we assembled the biosynthetic pathway of lovastatin in the methylotrophic yeast *P. pastoris* (Fig. 1) and successfully produced lovastatin from methanol (Liu et al. 2018). Nonetheless, gene dosages in the lovastatin-producing strains were all maintained at single-copy levels, and a titer of only 250.8 mg/L lovastatin was obtained via an optimal coculture strategy (Liu et al. 2018); this titer is still much lower than that of a native lovastatin-producing strain (Singh and Pandey 2013; Huang et al. 2017).

For the biosynthesis of chemicals in a heterologous host, increased gene dosage is commonly used to strengthen pathways (Billingsley et al. 2016). Nonetheless, overexpressed heterologous genes may impose overwhelming metabolic stress on the host cell, while excessive intracellular accumulation of bioactive compounds may be toxic to the cell (Wang et al. 2017). Fungal secondary-metabolite gene clusters often contain a gene encoding a protein to pump the produced compounds out of cells, which thereby acts as a self-resistance mechanism (Martín et al. 2005). Previously, efflux pump protein MlcE was identified in *Penicillium citrinum*, and lovastatin resistance was observed in *Saccharomyces cerevisiae* overexpressing *mlcE* (Ley et al. 2015). Because the lovastatin biosynthetic pathway had not yet been assembled in yeast (Ley et al. 2015), whether overexpression of *mlcE* could improve lovastatin production was not determined. Additionally, whether this protein functions as an efflux pump to transport intracellular lovastatin remains unclear.

This work was aimed at improving lovastatin production by means of methanol as a feedstock through strengthening of the biosynthetic pathway and screening for high gene dosage setups in three continuous modules. Moreover, to alleviate the toxicity or metabolic stress resulting from the product (lovastatin), a statin pump protein was identified in a lovastatin-producing strain of

A. terreus and overexpressed to discharge the intracellular lovastatin. Finally, the producing strains were evaluated in a 5-L bioreactor to confirm the effectiveness of the strategies.

Methods

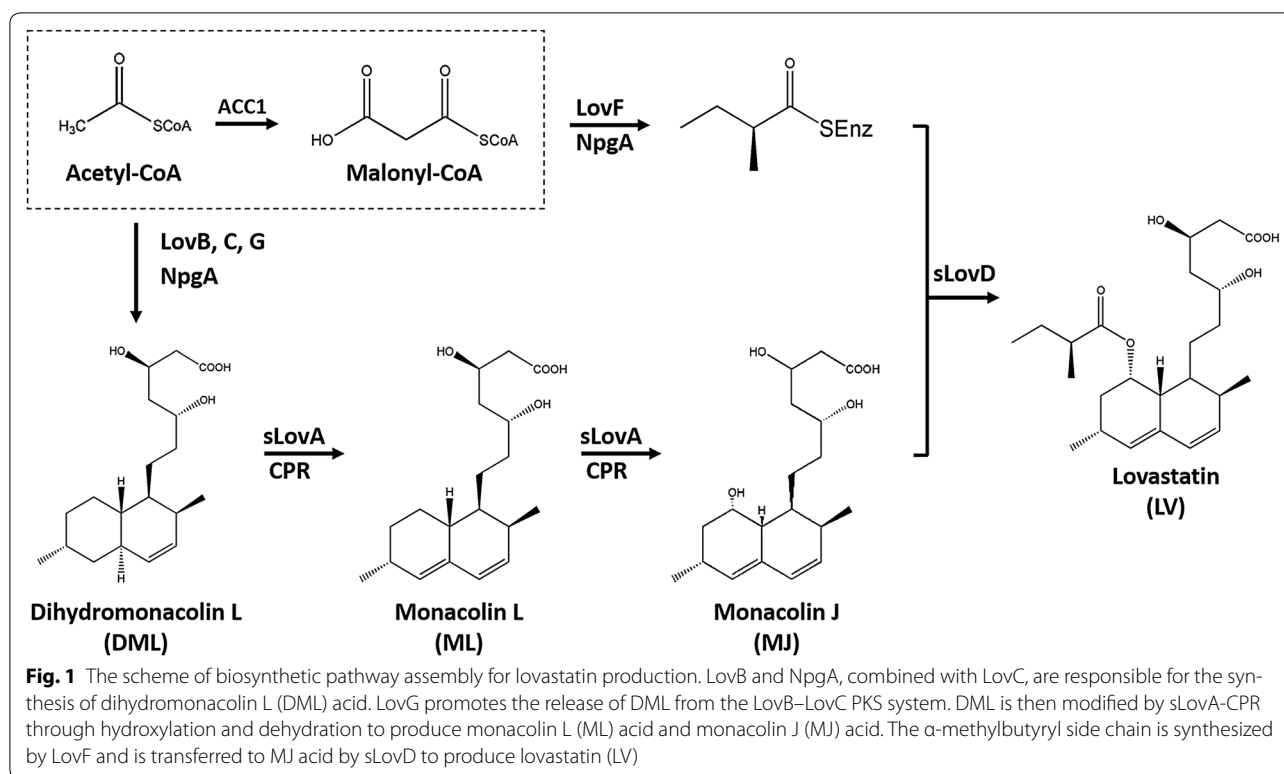
Strains, media, and cultivation conditions

Pichia pastoris GS115 (Invitrogen) served as a wild-type strain, grown at 30 °C in the YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] or the minimal YNB medium [1.34% (w/v) yeast nitrogen base without amino acids] supplemented with different carbon sources: 1% (w/v) glycerol (YNG medium) or 0.5% (v/v) methanol (YNM medium). *Escherichia coli* Top10 cells were used for plasmid propagation and were incubated at 37 °C in the Luria–Bertani (LB) medium [0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 0.5% (w/v) NaCl]. *A. terreus* (NIH 2624) was a source of the cDNA of *tapA* and was incubated at 28 °C in the YM medium [0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) tryptone, and 2% (w/v) glucose]. The solid medium was prepared by the addition of 2% agar into the liquid medium. The media were sterilized at 121 °C for 20 min; methanol was added separately, and a glucose solution was added separately after filtration sterilization. Antibiotics phleomycin D1 (Zeocin™), geneticin 418 sulfate, and hygromycin B, purchased from Yeasen Biotech Co., Ltd. (China), were used to screen the strains.

Construction of plasmids and strains

Plasmids pZ_BCGN, pK_sAR, and pG_sDF were constructed as previously described (Liu et al. 2018). pZ_BCGN was linearized by digestion with *BlnI* and was transferred into *P. pastoris* GS115 by electroporation in accordance with the manufacturer's instructions (Invitrogen). The yeast recombinants were screened on YPD plates containing Zeocin at 0.1, 0.5, 1.0, and 2.0 mg/mL to obtain dihydromonacolin L (DML)-producing strains (*P. p/DML*). pK_sAR was linearized by digestion with *SalI* and transferred into *P. p/DML_D#9* by the same methods, and the transformants were screened on YPD plates containing G418 at 0.25, 0.5, 1.0, and 2.0 mg/mL to obtain monacolin J (MJ)-producing strains (*P. p/MJ*). pG_sDF was linearized by *BlnI* digestion and transferred into *P. p/MJ_J#9*, and the transformants were screened on YPD plates containing hygromycin at 0.75, 1.5, 3.0, and 5.0 mg/mL to obtain lovastatin (LV)-producing strains (*P. p/LV*). The genotypes of the recombinant strains were verified by PCR analysis.

Oligonucleotides were synthesized by Suzhou Genewiz Biotech Co., Ltd. (China). Fungal genomic DNA was extracted using a Plant Genomic DNA Extraction Kit (Tiangen Biotech Co., Ltd., China). Commercialized



vector pGAP Z α for *P. pastoris* was linearized by digestion with *Bsp*T104I and *Kpn*I to delete the α -factor. The *tapA* gene was amplified by overlap PCR from the *A. terreus* NIH 2624 genome as a template, and the introns were removed. The multiple fragments and linearized vector were assembled by ClonExpress™ II One Step Cloning Kit (Vazyme Biotech Co., Ltd., China) to generate plasmid pZ_{P_{GAP}}-*tapA*. The same methods were applied to construct plasmids pZ_{P_{GAP}}-*gfp*, and pZ_{P_{GAP}}-*tapA-gfp*. These plasmids were linearized by *Bln*I digestion and transferred into *P. pastoris* GS115 to obtain strains GS-P_{GAP}-*gfp*, GS-P_{GAP}-*tapA-gfp*, and GS-P_{GAP}-*tapA*, respectively. Commercialized vector pPIC 6A for *P. pastoris* was linearized by digestion with *Eco*RI and *Sal*I; *tapA* was amplified and ligated with the linearized pPIC 6A to generate plasmid p6A_{P_{AOX1}}-*tapA*, which contains the expression cassette under the control of the *AOX1* promoter (P_{AOX1}). The expression vector was linearized by *Pme*I digestion and introduced into *P. p/LV_V#9* to obtain the *P. p/LV_V#9*-TapA strain. Plasmids and strains used in this study are listed in Additional file 1: Tables S1 and S2.

Phylogenetic, fluorescence-microscopic, and susceptibility analyses

The phylogenetic tree and bootstrap values were calculated by the neighbor-joining method implemented in

MEGA 7, according to the alignment of the amino acid sequences in the ClustalX software. The distance between branch points showed relatedness of the sequences. The amino acid sequence was BLASTed in the NCBI database. Proteins used to construct the phylogenetic tree are listed in Additional file 1: Table S3. Fluorescence microscopy of the constructed strains (GS_{P_{GAP}}-*tapA-gfp* and GS_{P_{GAP}}-*gfp*) was carried out under the inverted microscope DMI3000B (Leica) with a 100 \times oil immersion objective. Images were processed using the Leica application suite (version 2.8.1). For susceptibility assays on solid media, strains were grown in the YPD medium until optical density at 600 nm (OD₆₀₀) of 6, washed with sterile water, and then equal volumes of cell suspensions were spotted at four dilutions (OD₆₀₀ = 1, 0.1, 0.01, and 0.001) onto the YPD medium containing 0, 0.2, 0.5, or 1.0 mM lovastatin, respectively. The plates were cultured at 30 °C for 2 days and examined. For the susceptibility assay in a liquid medium, strains were grown in the YPD medium until OD₆₀₀ of 6 and washed with sterile water. The washed strains were inoculated in the YPD liquid medium containing 0, 0.5, or 1.0 mM lovastatin separately. The initial cell density was maintained at OD₆₀₀ = 0.5, and 2 mL of 50% (w/v) glucose was added every 24 h. Broth samples (1 mL) were collected at 24-h intervals (before and after the glucose feeding) to measure cell growth. Statistical analysis was performed on

three replicates, and the mean and standard deviation (SD) were calculated and displayed in the figures. Each experiment in this analysis section was conducted three times.

Measurement of concentrations of glucose, ethanol, and acetate

A biosensing analytical instrument (SBA-4AE) from the Biology Institute of Shan Dong Academy of Sciences was used to determine the concentrations of glucose and ethanol. Standard solutions of glucose (1 g/L) and ethanol (1 mL/L) were mixed and set to 100% in the calibration procedure. Acetic acid was analyzed by enzymatic assays (Megazyme, Ireland). Broth samples (1 mL) were collected and centrifuged at 4 °C and 12,000g for 2 min to obtain the supernatant, which was then diluted and assayed. Three replicates were analyzed; the mean with SD of the concentrations were calculated and displayed in figures. Measurement of the concentrations of glucose, ethanol, and acetate was conducted two times.

Fermentation in a shake flask

For shake flask culture, 20 µL of the strain (stored at - 80 °C) was inoculated into 2 mL of the YPD medium in a 20-mL serum bottle and grown at 30 °C for 2 days at 200 rpm for strain activation. Then, 200 µL of the cell broth was inoculated into 50 mL of the YPD medium in a 250-mL shake flask and grown overnight at 30 °C and 200 rpm to prepare the first seed culture. Next, 200 µL of the first seed culture was inoculated into 50 mL of the YPD medium in a 250-mL shake flask and grown overnight to OD₆₀₀ of 6 to prepare the second seed culture. A calculated amount of the second seed culture was centrifuged at 4 °C and 5000g for 10 min to harvest the cells, which were washed in the YNB medium with two further cycles of centrifugation (4 °C, 5000g). After that, the cells were resuspended in the YNB medium and inoculated into the YNM medium to final density for cultivation (OD₆₀₀=1.0). Methanol was added to the YNM medium at 0.5% (v/v) every 24 h. Broth samples (2 mL) were collected at 24-h intervals to measure the cell growth and metabolite concentrations. Statistical analysis was performed on three replicates; the mean with SD was calculated. Experiments for testing the production of the DML-, MJ- and LV-producing strains were conducted two times.

Extraction and identification of lovastatin and intermediates

Pichia pastoris culture broth was extracted with two volumes of ethyl acetate with vortex mixing for 5 min. The organic phase was evaporated under reduced pressure, and the residue was dissolved in methanol. The extracts

were analyzed by high-performance liquid chromatography (HPLC) on a C18 column (Kromasil™, Sweden, 250 mm × 4.6 mm × 5 mm, 100 Å spherical silica) with a gradient elution strategy at 1 mL/min and detection by UV absorbance at 238 nm (for monacolin L, monacolin J, and lovastatin) and by an evaporative light scattering detector operated at 85 °C and 2 mL/min (for DML). In both elution procedures, the mobile phase was composed of HPLC-grade H₂O containing 0.1% (v/v) acetic acid (solution A) and HPLC-grade acetonitrile (solution B). The sample was subjected to an elution gradient with a mobile phase comprising 35–85% (v/v) acetonitrile for 25 min followed by 85–100% (v/v) acetonitrile for 5 min. The final product, lovastatin, was confirmed by liquid chromatography with mass spectrometry (LC–MS; Agilent 1290 Infinity LC and 6230 TOF LC/MS) and nuclear magnetic resonance (NMR) spectroscopy (BRUKER-AM-400-spectro) of a freeze-dried sample dissolved in deuterated dimethyl sulfoxide for ¹H NMR analysis. The ¹H NMR results were then compared with previously reported data (Barriuso et al. 2011).

Bioreactor fermentation

This fermentation was conducted in a 5-L stirred-tank bioreactor (Shanghai Guoqiang Bioengineering Equipment Co., Ltd.) equipped with online sensors for dissolved oxygen (DO), pH, and temperature. The fermentation medium and control process were adapted from the *Pichia* Fermentation Process Guidelines (PFPG, Invitrogen). The strains were cultured overnight in YPD until the cell density reached OD₆₀₀ of 6 to prepare the first seed culture. The latter was inoculated into the MGY medium until it reached OD₆₀₀ of 6 and then served as the second seed culture; 300 mL of it was aseptically transferred into the bioreactor.

For bioprocess control, a batch phase involving the fermentation basal salt medium containing 40 g/L glycerol (PFPG, Invitrogen; Liu et al. 2018) was first conducted and maintained for approximately 18 h. A glycerol-fed batch phase was next carried out with a batch using 50% (w/v) glycerol for approximately 12 h, followed by a period of 0.5 h without any feeding, to allow the glycerol to be fully consumed. The methanol feed was implemented via a gradual increase in the methanol feeding rate from 2.4 to 24 g/h during the first 7.5 h, to allow the cells to adapt to methanol catabolism; after that, a constant rate of 24 g/h was maintained until the end of the experiment. DO levels reflect the remaining amount of a carbon source in a bioreactor; byproduct ethanol or acetate can also be utilized as carbon sources by *P. pastoris*. If these carbon sources highly accumulated in the broth, DO would remain at low levels because utilization of a carbon source consumes O₂. In contrast, when

the carbon sources were fully consumed, the DO would skyrocket. Following this strategy, we restrictedly controlled the feeding rate of glycerol and methanol according to DO levels and kept all carbon sources completely consumed. Broth samples (2 mL) were collected at 8-h intervals and analyzed twice to measure the cell growth and concentrations of the metabolites. The bioreactor fermentation experiments were conducted two times.

Results and discussion

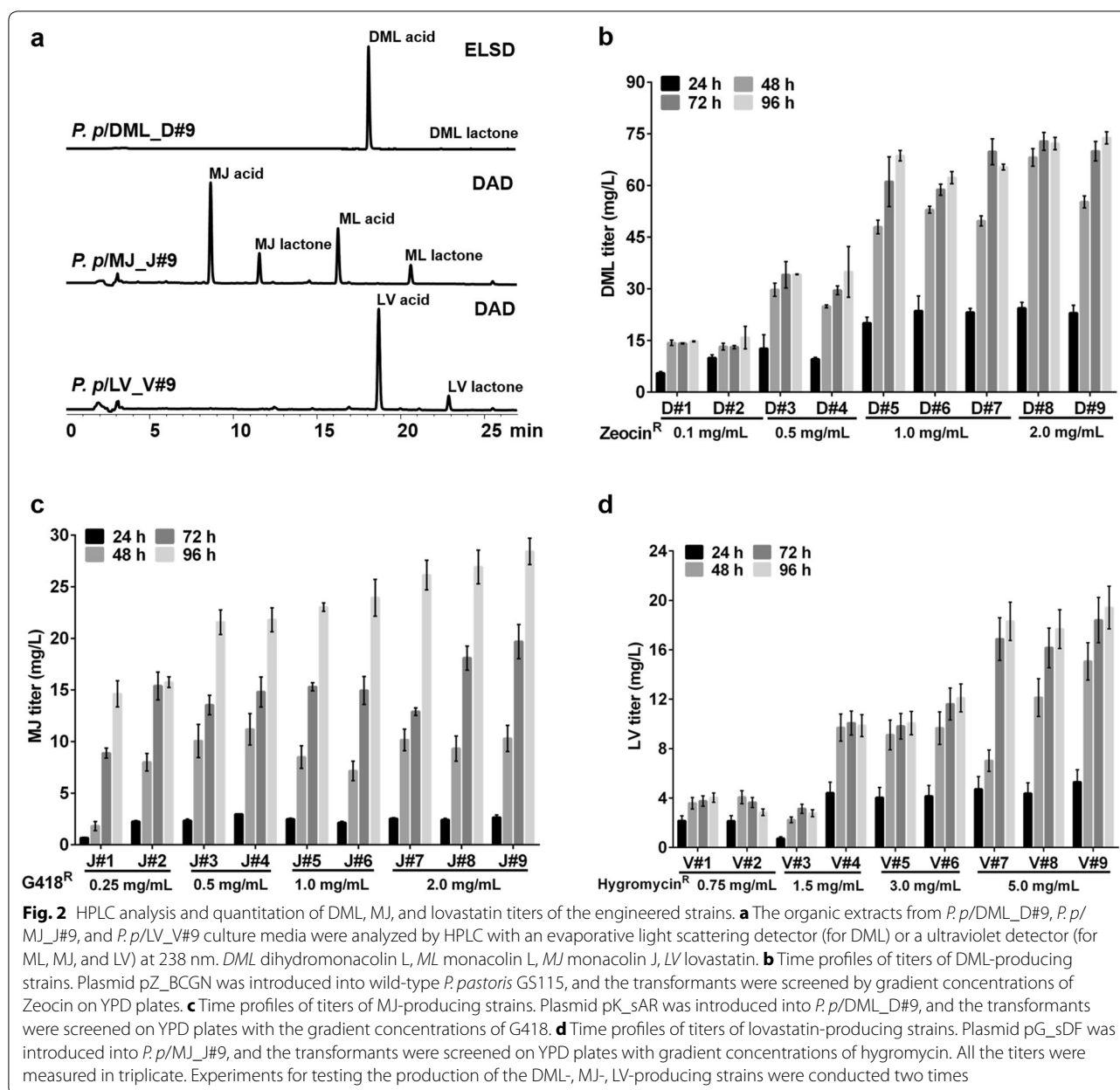
Assembly of an enhanced biosynthetic pathway of lovastatin

Plasmid pZ_BCGN was linearized, transferred into the wild-type strain, and employed to screen the microbes for DML-overproducing strains. As shown in Fig. 2a, b, strain *P. p/DML_D#9* grown at 2.0 mg/mL Zeocin produced the highest concentration of DML (73.8 ± 1.8 mg/L), and the production of DML increased as the Zeocin concentration was increased. Moreover, strain *P. p/DML_D#9* grew well in shake flask culture (Additional file 1: Figure S1a) and thus acted as a recipient strain for transformation with a second plasmid (pK_sAR) to generate MJ-overproducing strains. The resulting strain *P. p/MJ_J#9* grown at 2.0 mg/mL G418 showed the highest MJ titer (28.4 ± 1.3 mg/L; Fig. 2a, c), and its growth was not obviously affected (Additional file 1: Figure S1b). Similarly, the MJ titer also increased as the G418 concentration was increased; however, the obtained improvement was somewhat limited, while the intermediate monacolin L (ML) accumulated remarkably during culture (Fig. 2a). A third plasmid, pG_sDE, was then transferred into *P. p/MJ_J#9*, and the transformants were screened against different concentrations of hygromycin to find lovastatin-producing strains. Finally, a strain (*P. p/LV_V#9*) producing lovastatin at 19.4 ± 1.7 mg/L was obtained at 5.0 mg/mL hygromycin (Fig. 2a, d); this productivity was 592% higher than that of strain *P. p/LV_V#1* selected at 0.75 mg/mL hygromycin. The growth of these lovastatin-producing strains in shake flask culture appeared unaffected by the different concentration of antibiotics (Additional file 1: Figure S1c). Results of LC-MS and NMR analyses confirmed the module weight and structure of the product (lovastatin) produced by *P. p/LV_V#9* (Additional file 1: Figure S2). The pH values in the culture medium of lovastatin-producing strains were measured and are shown in Additional file 1: Figure S3; these levels similarly dropped from 5.5 to approximately 2.5, and final pH did not show a large difference among the lovastatin-producing strains. These results revealed that increasing the gene dosages by strengthening antibiotic stress during pathway assembly efficiently enhanced the production of certain compounds in lovastatin biosynthesis. Nevertheless, excessive expression of heterologous genes usually leads to severe

metabolic stress on the host, and the increased intracellular accumulation of target compounds may be harmful to the cell metabolism simultaneously. This problem may be inapparent during shake flask culture, but it will be conspicuous at a bioreactor fermentation level.

Enhancement of cell tolerance to lovastatin by identification and overexpression of a statin pump protein

Exogenous lovastatin was proved to inhibit yeast cell growth (Ley et al. 2015), whereas excessive intracellular accumulation of lovastatin may also be toxic and impose a metabolic burden on the host cell. Therefore, pumping intracellular lovastatin out of the *P. pastoris* cell through an efflux protein may relieve the toxicity effect and metabolic stress; as a consequence, the productivity of yeast cells may improve too. For this purpose, we conducted a BLAST search of the NCBI database for statin pump protein MlcE of *P. citrinum* (Ley et al. 2015); this search retrieved a hypothetical protein from *A. terreus* NIH2624, which was designated as TapA, consisted of 542 amino acid residues, and shared 69% sequence similarity with MlcE (Additional file 1: Figure S4). Phylogenetic analysis revealed that TapA and MlcE belong to a group of drug resistance proteins of the major facilitator superfamily (MFS): a large and diverse group of secondary transporters (Fig. 3a). The results indicated that TapA may be a statin efflux pump protein of the native lovastatin-producing strain of *A. terreus* and may improve the export efflux of lovastatin and, therefore, enhance the tolerance of *P. pastoris* to lovastatin. To determine the subcellular localization of TapA in engineered *P. pastoris*, we tagged TapA with green fluorescent protein (GFP) at its carboxylic terminus and expressed it as a single-copy gene. As a control, GFP was expressed alone using the same promoter and expression cassette of a single-copy gene. Fluorescence microscopy evaluation of the resulting strain, GS_P_{GAP}-*tapA-gfp*, revealed a ringlike distribution of the fluorescent protein at the periphery of the cells and the vacuole (Fig. 3b). By contrast, in the control strain, GS_P_{GAP}-*gfp*, a uniform cytoplasmic distribution of fluorescence was observed (Fig. 3b). The GFP-tagged TapA localized to the cytomembrane, indicating that TapA is a transmembrane protein, just as MlcE in *S. cerevisiae* (Ley et al. 2015). To test whether TapA could increase *P. pastoris* resistance to lovastatin, *tapA* was expressed under the control of constitutive promoter P_{GAP} in the wild-type strain. Strain GS_P_{GAP}-*tapA* was obtained and tested by serial dilution spotting on YPD plates supplemented with different concentration of lovastatin. As shown in Fig. 3c, the TapA-expressing strain showed increased resistance to lovastatin as compared with the wild-type strain.



To determine whether this TapA expression strategy could be applied to liquid fermentation, we cultured GS-*P_{GAP}-tapA* in the liquid YPD medium supplemented with increasing concentrations of lovastatin in shake flask culture. The growth of the wild-type strain was severely inhibited by lovastatin concentrations greater than 0.5 mM, whereas the growth of strain GS-*P_{GAP}-tapA* was affected only slightly (Fig. 3d). To avoid interference from the carbon source and byproduct formation, we next tested the concentrations of glucose, ethanol, and acetic acid in the lovastatin susceptibility assay. As shown in Fig. 4a, the glucose concentration in the medium of

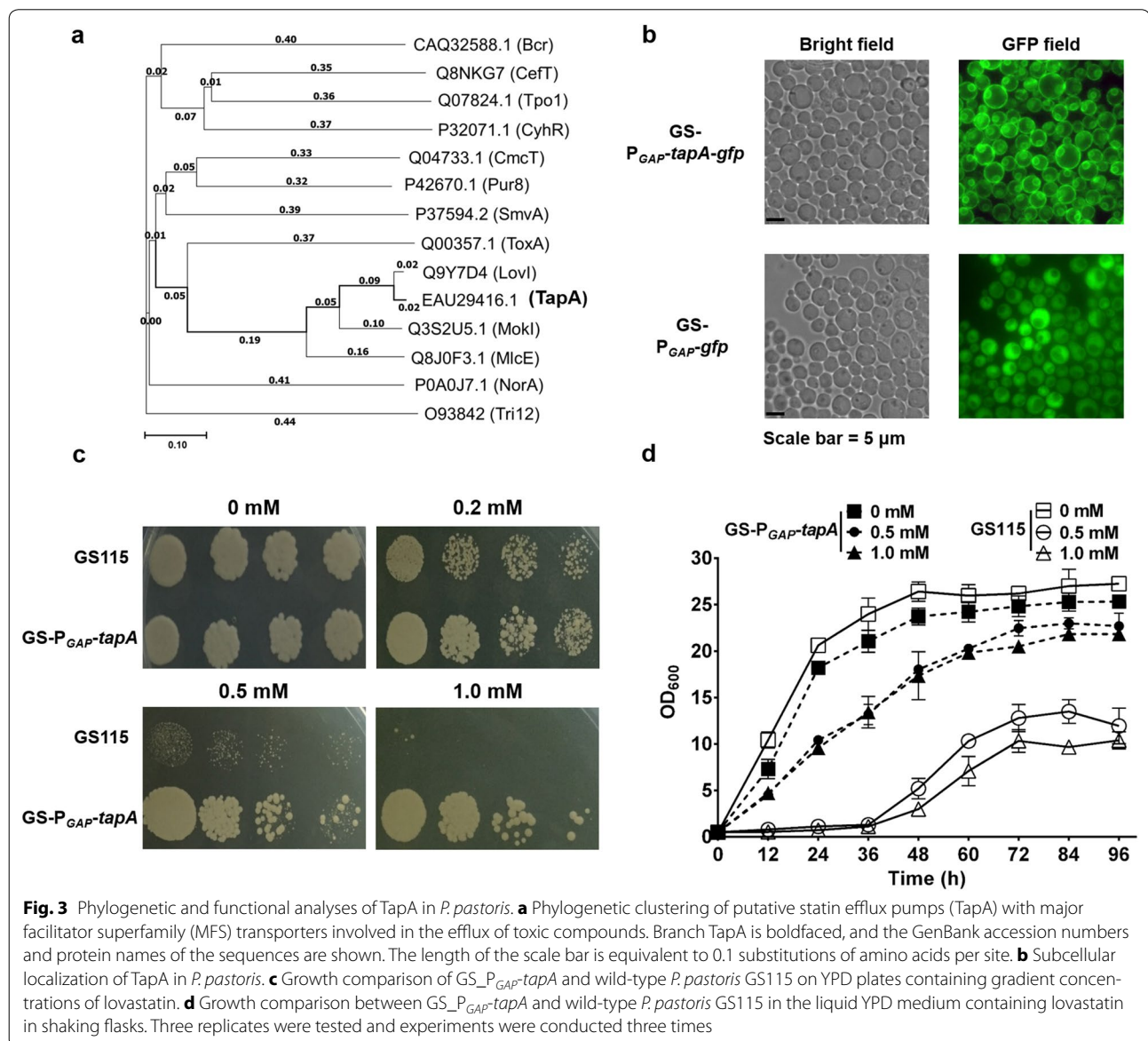
both strains showed the same trend without extracellular lovastatin feeding. When lovastatin was added at 0.5 or 1.0 mM, wild-type strain GS115 consumed less glucose, and residual glucose was more abundant in comparison with strain GS-*P_{GAP}-tapA*. These results confirmed that GS-*P_{GAP}-tapA* expressing the statin pump protein TapA is highly resistant to lovastatin; consequently, strain GS-*P_{GAP}-tapA* utilized more glucose and grew to higher cell density (Fig. 3d). Concentrations of ethanol and acetate were measured and are displayed in Fig. 4b, c. In the whole fermentation process, the concentrations of ethanol (Fig. 4b) and acetate (Fig. 4c) did not show a

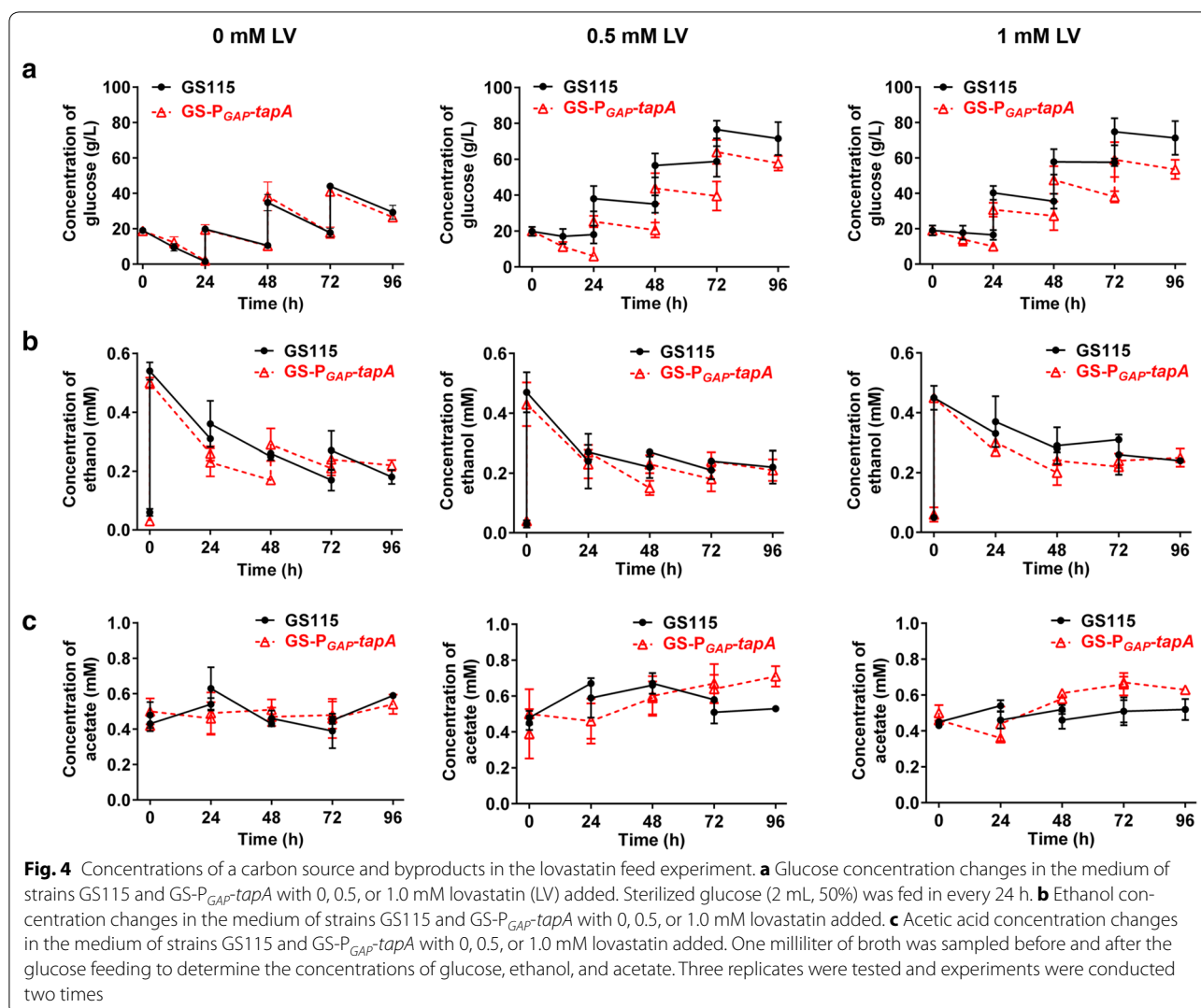
difference between strains GS115 and GS- $P_{GAP-tapA}$ whether lovastatin was added or not. Ethanol concentration stayed at approximately 0.2 mM, while acetic acid concentration stayed at approximately 0.4 mM. These results confirmed that formation of byproducts (ethanol or acetate) stayed at low levels and did not cause interference. These data implied that TapA-based resistance in *P. pastoris* may further improve the production of lovastatin in the *P. pastoris* cell factory.

Improvement in lovastatin production by coexpression of tapA

To test whether TapA could export the intracellular lovastatin and accordingly improve lovastatin production

in yeast, plasmid p6A- $P_{AOXI-tapA}$ was linearized and transferred into the multicopy strain *P. p/LV_V#9*, thus yielding a *tapA*-coexpressing strain *P. p/LV_V#9-TapA*. Both strains were cultured in the methanol medium (YNM), and a strain of *P. p/LV_SC* with all genes at single-copy levels (Liu et al. 2018) was cultured as a control. As shown in Fig. 5a, *P. p/LV_V#9-TapA* produced 32.9 ± 1.6 mg/L lovastatin, which was 69 and 520% higher than the productivity of *P. p/LV_V#9* and of the single-copy strain (Liu et al. 2018), respectively. The pH values in the medium of lovastatin-producing strains were nearly the same at the end of fermentation (Additional file 1: Figure S5). Compared with *P. p/LV_V#9*, *P. p/LV_V#9-TapA* accumulated less intracellular lovastatin



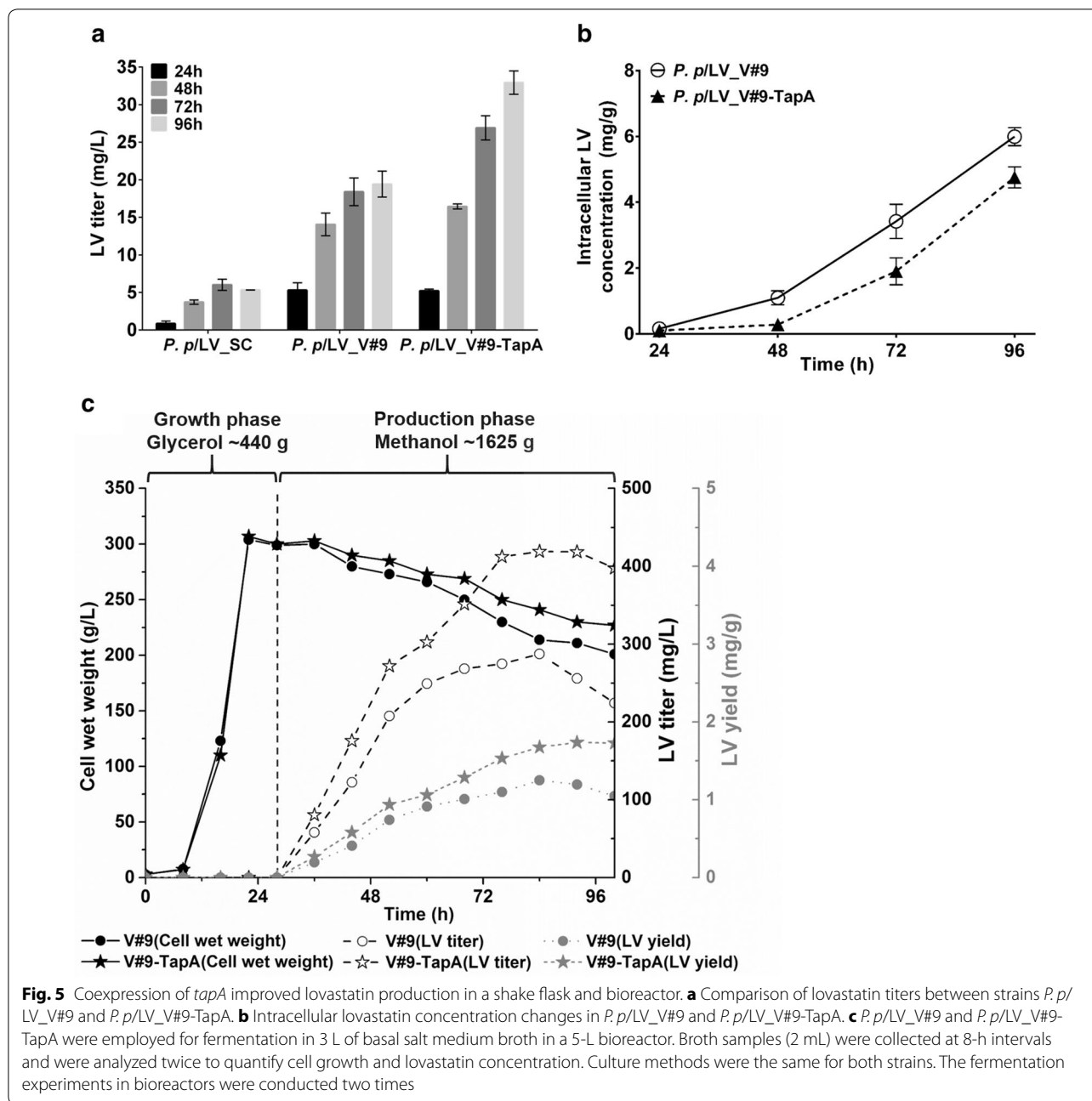


during cultivation (Fig. 5b), suggesting that TapA effectively pumped lovastatin out of the cell.

To further investigate the effects of TapA on lovastatin production, scaled-up experiments on both strains were conducted in a 5-L bioreactor. To this end, 3 L of basal salt medium broth (PFPG, Invitrogen) was used, pH was controlled at 4.5–5.0, and DO was maintained between 30 and 80% during the whole fermentation process (Additional file 1: Figure S6). *P. p/LV_V#9* produced 287.5 ± 2.0 mg/L lovastatin after 84 h (Fig. 5c), which was 267% higher than the productivity of the strain with a single copy of the biosynthetic genes (*P. p/LV_SC*), and even 15% higher than the productivity of the optimal pathway splitting and coculture strategy applied to the strains with a single copy of biosynthetic genes (Liu et al. 2018). Coexpression of TapA highly improved the production of lovastatin, to 419.0 ± 9.5 mg/L, after 84 h, which was 46% higher than the production by *P. p/LV_V#9*. In

addition, the yield per cell showed a similar trend, with a 39% increase. Because both strains expressed numerous heterologous genes, the cell wet weight decreased as the pathway startup was induced by methanol owing to metabolic stress from protein expression and biosynthesis of heterologous compounds. Nevertheless, strain *P. p/LV_V#9-TaP* carrying the statin pump protein grew better than *P. p/LV_V#9* did (Fig. 5c), indicating that lovastatin efflux by TapA partially relieved the inhibition of cell growth and effectively improved lovastatin production.

Although *P. pastoris* has been recognized and widely used as a heterologous protein expression system (Ahmad et al. 2014; Yang and Zhang 2017), application of this classic microbe to reproduce medicinal chemicals via complicated biosynthetic pathways from methanol has seldom been reported. This work shows a strategy of de novo production of lovastatin from methanol in the methylotrophic yeast *P. pastoris* as a host. Considering



its particular advantages in methanol utilization and protein expression, *P. pastoris* is expected to be developed into a promising microorganism for the synthesis of bulk pharmaceuticals from a potentially renewable and low-cost feedstock, methanol. Meanwhile, a lovastatin-transporting membrane protein was identified in the industrial host *A. terreus*. Although a similar efflux protein has been recently reported to improve yeast resistance to external lovastatin (Ley et al. 2015), a concrete function of the pump protein was not completely revealed. Here, the ability of this statin pump protein to

export intracellular lovastatin was confirmed via coexpression in a lovastatin-producing strain, and its capacity for improving lovastatin production was verified as well. Nevertheless, lovastatin production in this study is still far below the titer of the industrial lovastatin-producing *A. terreus* strain, which generates 14.26 mM (~6.1 g/L) lovastatin after 8 days of fermentation (Huang et al. 2017). The highest heterologous production of lovastatin was 250.8 mg/L in a *P. pastoris*-*P. pastoris* coculture system in our previous work (Liu et al. 2018). Although we improved lovastatin production to 419.0 ± 9.5 mg/L after

84 h here, further research is necessary to determine whether this approach is suitable for industrial production of lovastatin, and this work is still an academic study. Consequently, extensive research into systematic metabolic engineering and bioprocess development needs to be conducted. For instance, *tapA* was originally cloned from *A. terreus*, and codons could be optimized for higher expression of TapA in *P. pastoris*. Pathway splitting and coculture could be combined with an increase in gene dosage and modification and expression of a statin pump protein in the future, and this strategy may further improve lovastatin production. The bioreactor fermentation process here is derived from the manual for basal protein expression in *P. pastoris* (Invitrogen). Bioprocess development, such as control of the methanol feeding rate or cell growth, will certainly improve lovastatin production.

Conclusions

A gene dosage increase driven by antibiotic stress in pathway assembly efficiently enhanced lovastatin production from methanol in *P. pastoris*. A statin pump protein was identified in a native lovastatin-producing microbe, and membrane localization of the new pump in engineered *P. pastoris* was confirmed. Coexpression of the pump protein enhanced cell resistance to lovastatin, strengthened the intracellular lovastatin efflux, and improved the production of lovastatin in *P. pastoris*. These results also proved that *P. pastoris* is a promising chassis host for heterologous biosynthesis of natural products from the one-carbon substrate, methanol.

Additional file

Additional file 1. Additional figures and tables.

Abbreviations

P. pastoris: *Pichia pastoris*; DML: dihydromonacolin L; ML: monacolin L; MJ: monacolin J; LV: lovastatin.

Authors' contributions

MC conceived the project and supervised the research. YL, CB, and MC were responsible for project planning and experimental design. YL and CB conducted all the experiments. QX and CB participated in the construction of the strains. YL and JY participated in the bioreactor fermentation experiments. YL, CB, and MC analyzed the data. YL wrote the manuscript, MC revised and enhanced the manuscript. All the coauthors contributed to the scientific discussion. All authors read and approved the final manuscript.

Author details

¹ State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China. ² Shanghai Collaborative Innovation Center for Biomanufacturing, Shanghai 200237, China.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated during this study are included in this article.

Consent for publication

The authors approved the consent for publishing the manuscript.

Ethics approval and consent to participate

We declare that this paper does not report any data collected from humans or animals.

Funding

This work was supported by Fundamental Research Funds for the Shanghai Science and Technology Innovation Action Plan (Grant number 17JC1402400) and the Talent Program of the School of Biotechnology at East China University of Science and Technology.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 3 February 2018 Accepted: 28 March 2018

Published online: 17 May 2018

References

- Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl Microbiol Biotechnol* 98:5301–5317
- Barriuso J, Nguyen DT, Li JW, Roberts JN, MacNevin G, Chaytor JL, Marcus SL, Vederas JC, Ro DK (2011) Double oxidation of the cyclic nonaketide dihydromonacolin L to monacolin J by a single cytochrome p450 monooxygenase, lovA. *J Am Chem Soc* 133:8078–8081
- Bennett RK, Steinberg LM, Chen W, Papoutsakis ET (2018) Engineering the bioconversion of methane and methanol to fuels and chemicals in native and synthetic methylotrophs. *Curr Opin Biotechnol* 50:81–93
- Billingsley JM, DeNicola AB, Tang Y (2016) Technology development for natural product biosynthesis in *Saccharomyces cerevisiae*. *Curr Opin Biotechnol* 42:74–83
- Campbell CD, Vederas JC (2010) Biosynthesis of lovastatin and related metabolites formed by fungal iterative pks enzymes. *Biopolymers* 93:755–763
- Cregg JM, Cereghino JL, Shi J, Higgins DR (2000) Recombinant protein expression in *Pichia pastoris*. *Mol Biotechnol* 16:23–52
- Galanie S, Thodey K, Trenchard LJ, Interrante MF, Smolke CD (2015) Complete biosynthesis of opioids in yeast. *Science* 349:1095–1100
- Huang X, Liang Y, Yang Y, Lu X (2017) Single-step production of the simvastatin precursor monacolin J by engineering of an industrial strain of *Aspergillus terreus*. *Metab Eng* 42:109–114
- Kennedy J, Auclair K, Kendrew SG, Park C, Vederas JC, Hutchinson CR (1999) Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* 284:1368–1372
- Ley A, Coumou HC, Frandsen RJN (2015) Heterologous expression of MlcE in *Saccharomyces cerevisiae* provides resistance to natural and semi-synthetic statins. *Metab Eng Commun* 2:117–123
- Liu Y, Tu X, Xu Q, Bai C, Kong C, Liu Q, Peng Q, Zhou X, Zhang Y, Cai M (2018) Engineered monoculture and co-culture of methylotrophic yeast for de novo production of monacolin J and lovastatin from methanol. *Metab Eng* 45:189–199
- Martin JF, Casqueiro J, Liras P (2005) Secretion systems for secondary metabolites: how cells send out messages of intercellular communication. *Curr Opin Microbiol* 8:282–293
- Price JV, Chen L, Whitaker WB, Papoutsakis E, Chen W (2016) Scaffoldless engineered enzyme assembly for enhanced methanol utilization. *Proc Natl Acad Sci USA* 113:12691–12696
- Räuchle K, Plass L, Wernicke HJ, Bertau M (2016) Methanol for renewable energy storage and utilization. *Energy Technol* 4:193–200

- Ro DK, Paradise EM, Ouellet M, Fisher KJ et al (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440:940–943
- Schwarzshans JP, Luttermann T, Geier M, Kalinowski J, Friehs K (2017) Towards systems metabolic engineering in *Pichia pastoris*. *Biotechnol Adv* 35(6):681–710
- Singh SK, Pandey A (2013) Emerging approaches in fermentative production of statins. *Appl Biochem Biotechnol* 171(4):927–938
- Vogl T, Hartner FS, Glieder A (2013) New opportunities by synthetic biology for biopharmaceutical production in *Pichia pastoris*. *Curr Opin Biotechnol* 24:1094–1101
- Wang F, Lv X, Xie W, Zhou P, Zhu Y, Yao Z, Yang C, Yang X, Ye L, Yu H (2017) Combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase to improve production in *Saccharomyces cerevisiae*. *Metab Eng* 39:257–266
- Whitaker WB, Sandoval NR, Bennett RK, Fast AG, Papoutsakis ET (2015) Synthetic methylotrophy: engineering the production of biofuels and chemicals based on the biology of aerobic methanol utilization. *Curr Opin Biotechnol* 33:165–175
- Xie X, Watanabe K, Wojcicki WA, Wang CC, Tang Y (2006) Biosynthesis of lovastatin analogs with a broadly specific acyltransferase. *Chem Biol* 13:1161–1169
- Xu W, Chooi YH, Choi JW, Li S, Vederas JC, Da Silva NA, Tang Y (2013) LovG: the thioesterase required for dihydromonacolin L release and lovastatin nonaketide synthase turnover in lovastatin biosynthesis. *Angew Chem Int Ed* 52:6472–6475
- Yang Z, Zhang Z (2017) Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris*: a review. *Biotechnol Adv*. <https://doi.org/10.1016/j.biotechadv.2017.11.002> (in press)

Submit your manuscript to a SpringerOpen[®] journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com
