

# Improving simvastatin bioconversion in *Escherichia coli* by deletion of *bioH*

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## Abstract

Simvastatin is an important cholesterol lowering compound and is currently synthesized from the natural product lovastatin via multistep chemical synthesis. We have previously reported the use of an *Escherichia coli* strain BL21(DE3)/pAW31 as the host for whole-cell biocatalytic conversion of monacolin J acid to simvastatin acid. During fermentation and bioconversion, unknown *E. coli* enzyme(s) hydrolyzed the membrane permeable thioester substrate dimethylbutyryl-*S*-methyl mercaptopropionate (DMB-*S*-MMP) to the free acid, significantly decreased the efficiencies of the whole-cell bioconversion and the downstream purification steps. Using the Keio K-12 Single-Gene Knockout collection, we identified BioH as the sole enzyme responsible for the observed substrate hydrolysis. Purification and reconstitution of *E. coli* BioH activity *in vitro* confirmed its function. BioH catalyzed the rapid hydrolysis of DMB-*S*-MMP with  $k_{\text{cat}}$  and  $K_{\text{m}}$  values of  $260 \pm 45 \text{ s}^{-1}$  and  $229 \pm 26 \mu\text{M}$ , respectively. This is in agreement with previous reports that BioH can function as a carboxylesterase towards fatty acid esters. YT2, which is a  $\Delta\text{bioH}$  mutant of BL21(DE3), did not hydrolyze DMB-*S*-MMP during prolonged fermentation and was used as an alternative host for whole-cell biocatalysis. The rate of simvastatin acid synthesis in YT2 was significantly faster than in BL21(DE3) and 99% conversion of 15 mM simvastatin acid in less than 12 h was achieved. Furthermore, the engineered host required significantly less DMB-*S*-MMP to be added to accomplish complete conversion. Finally, simvastatin acid synthesized using YT2 can be readily purified from fermentation broth and no additional steps to remove the hydrolyzed dimethylbutyryl-*S*-mercaptopropionic acid is required. Together, the proteomic and metabolic engineering approaches render the whole-cell biocatalytic process more robust and economically attractive.

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## 1. Introduction

Simvastatin is an effective cholesterol lowering compound and is one of the best selling drugs in the world (Freeman, 2006; Tobert, 2003). It is a semisynthetic compound obtained from the natural product lovastatin, which is produced by *Aspergillus terreus* as a secondary metabolite (Endo, 1980; Hendrickson et al., 1999; Kennedy et al., 1999). Optimization of the multistep, chemical synthesis of simvastatin starting from lovastatin is of intense interest for the past two decades (Askin et al., 1991; Hoffman et al., 1986). We recently characterized a novel acyltransferase,

LovD, encoded in the lovastatin gene cluster (Xie et al., 2006). LovD catalyzes the last step of lovastatin biosynthesis and is responsible for transferring the 2-methylbutyrate side chain from the megasynthase LovF to the immediate biosynthetic precursor, monacolin J (MJ) acid (Kennedy et al., 1999). We demonstrated that LovD displays broad substrate specificity towards the decalin core, the thioester acyl unit and the thioester acyl carrier. Using an *Escherichia coli* strain overexpressing LovD and a cell-membrane permeable thioester dimethylbutyryl-*S*-methyl mercaptopropionate (DMB-*S*-MMP) (Fig. 1A), we developed a whole-cell biocatalytic process that can convert MJ acid to simvastatin acid in one step with high yields (Xie and Tang, 2007). The fermentation process can be an economically competitive alternative to the current synthetic routes.

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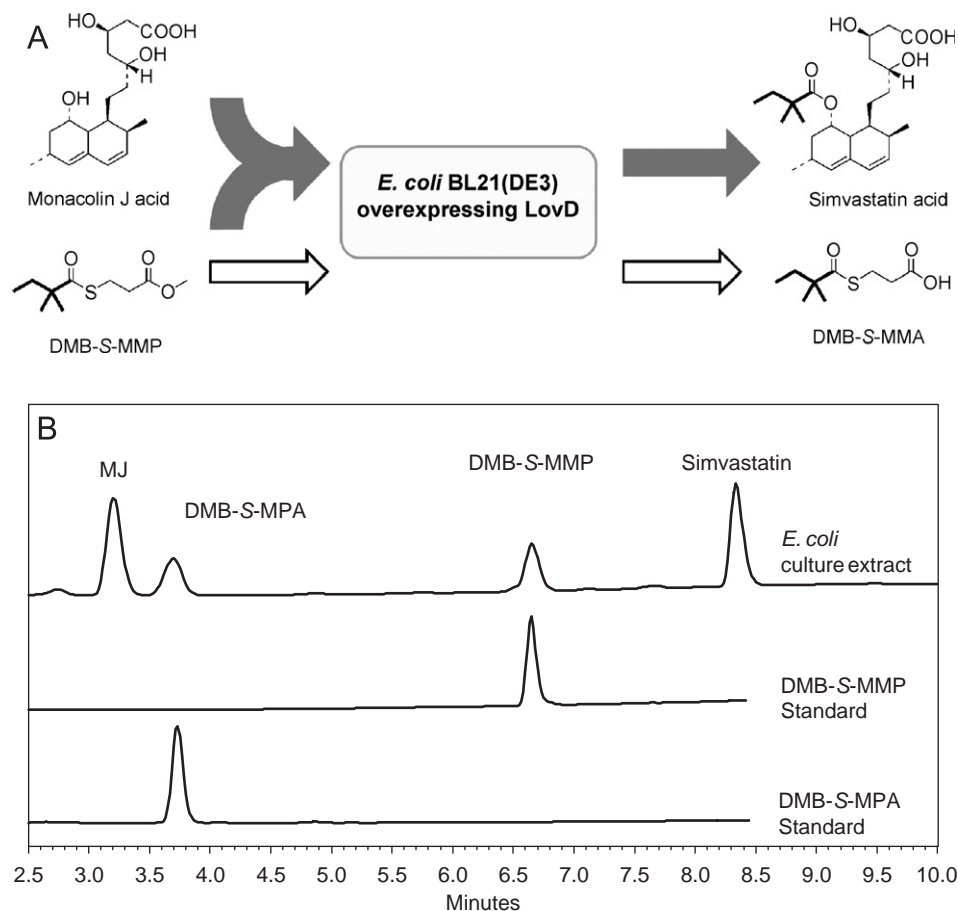


Fig. 1. (A) Whole-cell biocatalytic conversion of simvastatin acid from monacolin J acid and DMB-S-MMP. The *E. coli* strain overexpresses the acyltransferase LovD. Hydrolysis of DMB-S-MMP to yield DMB-S-MPA is a competing reaction. (B) Top trace: typical profile of reactants and products from a high-cell density bioconversion experiment demonstrating the extent of DMB-S-MMP hydrolysis. Bottom traces: standards for DMB-S-MMP and DMB-S-MPA. The spectra were collected at 238 nm.

The thioester DMB-S-MMP is an integral component of simvastatin bioconversion. It is among the most catalytically efficient acyl donors examined for the acyltransfer reaction, while being the least expensive to synthesize. One significant drawback associated with this compound is hydrolysis of the methyl ester bond in DMB-S-MMP that yields dimethylbutyryl mercaptopropionic acid (DMB-S-MPA) (Fig. 1). The hydrolysis reaction is enzymatic because no degradation was observed in the absence of *E. coli*, and is dramatically elevated during high cell-density fermentation (Fig. 1B). The side reaction is undesirable for three reasons: (1) hydrolysis of the substrate depletes DMB-S-MMP available for LovD-catalyzed transacylation, requiring the thioester to be added in high molar excess and to be replenished frequently during fermentation; (2) since DMB-S-MPA is less efficient compared to DMB-S-MMP as a dimethylbutyrate donor (~10-fold slower) (Xie and Tang, 2007), accumulation of the more soluble DMB-S-MPA can serve as a competing acyl substrate for LovD. This effectively decreases the reaction velocity and has been demonstrated *in vitro* using purified LovD. Therefore, the overall duration of the bioconversion

is unnecessarily prolonged; and (3) the carboxylic acid moiety of DMB-S-MPA interferes with purification of simvastatin acid from the culture medium at completion of the bioconversion. Both compounds precipitate from the culture broth upon acidification of the medium and additional separation steps are required before crystallization of simvastatin. While DMB-S-MPA can be removed by washing the filtrate with excessive amount of water, an appreciable amount of simvastatin acid were lost during the washing steps, resulting in lower overall recovery.

Eliminating the undesirable hydrolysis side reaction can therefore improve the economics of the whole-cell biocatalytic process and the downstream purification steps. The most immediate goal is to pinpoint the enzyme(s) responsible for the undesirable side reaction. Here, we report the identification of BioH as the *E. coli* carboxylesterase that hydrolyzes DMB-S-MMP into DMB-S-MPA. By constructing a  $\Delta bioH$  derivative of the LovD overexpression strain, we completely eliminated the competing reaction and further improved the robustness of the whole-cell biocatalytic synthesis of simvastatin acid.

## 2. Materials and methods

### 2.1. Materials, strains and plasmids

Monacolin J and DMB-S-MMP were prepared as described previously (Xie and Tang, 2007). All reagents were purchased from standard sources. The BL21(DE3) strain [ $F^- omp hsdS_B (r_B^- m_B^-) gal dem \lambda(DE3)$ ] was obtained from Novagen. The Keio collection was obtained from the National Institute of Genetics, Japan (Baba et al., 2006). The single-gene knockout mutants were derived from the BW25113 strain [ $rrnB3 DElacZ4787 hsdR514 DE(araBAD)567 DE(rhaBAD)568rph-I$ ]. WA837 ( $r_B^-, m_B^+, gal met$ ), an *E. coli* B strain that is restriction-minus and modification-plus was obtained from The Coli Genetic Stock Center (CGSC) (Wood, 1966). The plasmids pAW31 ( $kan^r$ ) and pXK8 ( $kan^r$ ) were derived from pET28a and contain the *lovD* gene from *A. terreus* and the *bioH* gene from *E. coli*, respectively.

### 2.2. Whole-cell-based hydrolysis assay

The selected 57 mutants from the Keio collection together with BW25113, BL21(DE3) and BL21(DE3)/pAW31 were grown to saturation in a 96-well deep well plate (Fisher Scientific) in 1 ml LB media at 37 °C. Neat DMB-S-MMP (5  $\mu$ l) was added to each culture with a final concentration of 20 mM. After shaking (20 °C, 300 rpm) for 10 h, each culture was extracted with an equal volume of ethyl acetate (EA)/1% acetic acid (AcOH). The organic phase was dried, redissolved in 20  $\mu$ l acetonitrile (CH<sub>3</sub>CN), and 1  $\mu$ l was spotted on a TLC plate (silica gel 60 F254). The TLC plates were developed with 20% EA in hexane and visualized with iodine.

Analysis of the compounds were also performed with HPLC using an analytical C<sub>18</sub> column (Alltech Apollo 5u, 150 mm  $\times$  4.6 mm); linear gradient: 60% CH<sub>3</sub>CN in water (0.1% trifluoroacetic acid [TFA]) to 95% CH<sub>3</sub>CN in water (0.1% TFA) over 5 min, 95% CH<sub>3</sub>CN in water (0.1% TFA) for 10 min, with a flow rate of 1 ml/min. HPLC retention times ( $t_R$ ) were as follows: MJ lactone form: 3.40 min; DMB-S-MPA: 3.82 min; DMB-S-MMP: 6.80 min; simvastatin lactone form: 8.45 min. Both MJ and simvastatin acids were lactonized before HPLC analysis.

### 2.3. Purification of *bioH* and enzymatic assay

The *bioH* gene was amplified from *E. coli* genomic DNA by PCR with flanking restriction sites *Nde*I and *Eco*RI using the primers 5'-AACATATGAATAACATCTGGTG GCA-3' and 5'-AAGAATTCTACACCCTCTGCTTCAA CG-3'. The gene cassette was digested and ligated into pET28a to yield the expression construct pXK8. The *E. coli* BL21(DE3) strain transformed with pXK8 was grown in LB medium at 37 °C to an OD<sub>600</sub> of 0.5, at which time 0.1 mM IPTG was added to the culture and expression was performed at 20 °C for 16 h. Cells were resuspended in Buffer A (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM

EDTA) and lysed with sonication. Purification of BioH was facilitated by the N-terminal 6  $\times$  His tag and Ni-NTA resin (Qiagen). Nearly pure (>95%) BioH was eluted in Buffer A containing 250 mM imidazole, which was buffer exchanged into Buffer A, concentrated, aliquoted and flash frozen.

The hydrolysis assays were performed at room temperature in 50 mM HEPES, pH 7.9. The thioester substrate DMB-S-MMP was added to final concentration between 0.1 and 1.0 mM. To facilitate solubilization of DMB-S-MMP, DMSO was added to a final concentration of 10% for all the samples. The reaction was initiated with the addition of BioH (0.01  $\mu$ M) and quenched at desired time points (10, 20, and 30 min) by adding equal volume of EA/1% AcOH. The organic phase was separated, dried and redissolved in 20  $\mu$ l of ACN and analyzed with HPLC. The conversions of DMB-S-MMP to DMB-S-MPA were quantified by integration of the peaks at 234 nm. Comparison of the BioH catalytic efficiency towards the three dimethylbutyryl thioesters was performed at 1 mM substrate concentration and 10 nM BioH concentration.

### 2.4. P1 transduction

Following standard protocols (Miller, 1992), P1 transduction was used to construct the  $\Delta bioH$  deletion mutant of BL21(DE3). Because of the different restriction system between *E. coli* K strain (BW25113) and B strain (BL21), the B strain WA837 ( $r_B^-, m_B^+$ ) was used as an intermediate host for transduction (Dien et al., 2001). The  $\Delta bioH::FRT-kan-FRT$  marker was first transduced from JW3375 to WA837 to yield YT0 (WA837  $\Delta bioH::FRT-kan-FRT$ ). Using BL21(DE3) as a recipient and YT0 as a donor, strain YT1 (BL21  $\Delta bioH::FRT-kan-FRT \lambda(DE3)$ ) was constructed. The YT1 strain was transformed with the helper plasmid pCP20 which contains a temperature sensitive replication and thermally inducible FLP gene (Datsenko and Wanner, 2000). Removal of the *kan* marker to yield YT2 ((BL21  $\Delta bioH::FRT \lambda(DE3)$ ) followed published procedures (Datsenko and Wanner, 2000).

PCR was used to verify the genetic changes of YT2. Three primers were designed. The primers B1: 5'-TGA-CGGCTTCGCTATCCCAT-3'; B2: 5'-TACACCCTCTG-CTTCAACG-3'; and B3: 5'-GCTGGATTGTTTCGCC-GATC-3' anneal to the upstream gene *yhqA*, the 3' end of *bioH* that was left intact, and the downstream gene *gntX*, respectively. The expected products for PCR reaction using YT2 genomic DNA as template are: B1/B2: 602 bp; B1/B3: 1002 bp. The expected products for PCR reaction using BL21(DE3) genomic DNA as template are: B1/B2: 1271 bp; B1/B3: 1771 bp. Only the expected PCR products were observed in each reaction.

### 2.5. Whole-cell biocatalysis

Whole-cell catalytic synthesis of simvastatin acid from MJ acid and DMB-S-MMP were performed as described

(Xie and Tang, 2007). The *E. coli* BL21(DE3)/pAW31 and YT2/pAW31 strains were cultured side-by-side for comparison. A single colony of the freshly transformed strains was used to inoculate a 5 ml LB culture supplemented with 35 mg/l kanamycin and grown overnight at 37 °C. The next morning, 100 µl of the overnight culture was inoculated into 50 ml cultures containing LB broth, F1 minimal medium and F1 medium supplemented with 0.15 mg/l biotin. Growth rates were monitored by periodically measuring the OD<sub>600</sub> reading. When OD<sub>600</sub> reached ~0.5, 100 µM IPTG was added to the culture and expression of *lovD* was performed at 20 °C for 16 h. To mimic high-density fermentation conditions, the cells were concentrated 10-fold before addition of substrates. Typically, a 14 ml aliquot of the culture was transferred to a 15 ml centrifuge tube and the cells were collected by centrifugation (4 °C, 4000 g, 10 min). The cell pellet was gently resuspended in 1316 µl of the supernatant, followed by addition of 84 µl of a MJ acid stock solution (250 mM in H<sub>2</sub>O) (final concentration 15 mM). The concentrated culture was then separated into seven 200 µl samples and 1.2 µl of pure DMB-S-MMP was added to each sample (final concentration ~25 mM). The culture was then shaken at 300 rpm at room temperature. At each time point, a total extraction was performed by adding 10 µl 20% SDS to lyse the cells, followed by liquid–liquid extraction with 500 µl EA/1% AcOH. The organic phase was removed, evaporated, and redissolved in 50 µl ACN for HPLC analysis. For the BL21(DE3) sample, an additional 1.2 µl aliquot of DMB-S-MMP was added after 12 h to replenish the hydrolyzed substrates.

### 3. Results and discussion

#### 3.1. Identification of *bioH* as the DMB-S-MMP esterase

We first aimed to identify the *E. coli* enzyme that is responsible for the observed hydrolysis of DMB-S-MMP into DMB-S-MPA during fermentation. When different acyl carriers such as dimethylbutyryl-*S*-ethyl mercaptopropionate (DMB-S-EMP) and dimethylbutyryl-*S*-methyl thioglycolate (DMB-S-MTG) were used as the thioester substrates, we observed the corresponding hydrolyzed carboxylic acids in the fermentation broth (data not shown). This suggests the responsible enzyme is an esterase or hydrolase with relaxed substrate specificity towards ester functionalities.

We used a high-throughput approach to identify the responsible enzyme, utilizing the *E. coli* K-12 in-frame single-gene knockout mutant library (Keio Collection) (Baba et al., 2006). We reasoned that a mutant strain that is unable to hydrolyze DMB-S-MMP is directly attributed to the specific single-gene deletion. Examination of *E. coli* genome annotations (Blattner et al., 1997) reveals 23 esterases/esterase-like enzymes, 94 hydrolases/hydrolase-like enzymes, and 16 acyltransferases (133 total candidate genes). Enzymes with confirmed activities and substrates

that are unlikely to be involved in DMB-S-MMP hydrolysis were not examined in the first round of assays. The list of 57 BW25113 mutant strains examined is shown in Table 1.

The mutants, wild-type BW25113 and BL21(DE3) were grown to saturation in LB broth in a 96-well deep well plate. We added 5 µl of neat DMB-S-MMP to each culture (1 ml) and the plate was shaken vigorously for 10 additional hours at room temperature. The organic extract from each culture analyzed by thin layer chromatography (TLC) using a mobile phase (20% ethyl acetate in hexane) that enabled separation of DMB-S-MMP and DMB-S-MPA (Fig. 2A). The wild-type BW25113 (lane 58) and BL21(DE3) (lane 59) each showed a comparable level of substrate hydrolysis and accumulation of DMB-S-MPA. All of the mutants examined displayed hydrolytic activity towards DMB-S-MMP, except  $\Delta bioH$  (lane 56, strain JW3357). This surprising finding suggests that BioH (O'Regan et al., 1989), which is involved in the biosynthesis of biotin (vitamin H), may be the sole enzyme responsible for the observed hydrolysis *in vivo*. Additional examination using HPLC further confirmed that DMB-S-MPA cannot be detected in the organic extract of the  $\Delta bioH$  mutant, while nearly 30% were hydrolyzed in *bioH*<sup>+</sup> strains (Fig. 2B).

#### 3.2. Verification of *bioH* properties *in vitro*

To prove that BioH is directly involved in hydrolyzing DMB-S-MMP during fermentation, we cloned the *bioH* gene and expressed it as an *N*-his-tagged protein from BL21(DE3) (Tomczyk et al., 2002). The protein was purified to homogeneity using Ni-NTA affinity chromatography with a final yield of 9 mg/l. The catalytic properties of BioH towards DMB-S-MMP were assayed and the

Table 1  
List of *E. coli* BW25113 mutants screened in this work

No.	Gene deletion	No.	Gene deletion	No.	Gene deletion	No.	Gene deletion
1	<i>ΔgloB</i>	16	<i>ΔyegU</i>	31	<i>ΔpaaI</i>	46	<i>ΔyidA</i>
2	<i>ΔmhpC</i>	17	<i>ΔlpxL</i>	32	<i>ΔyegX</i>	47	<i>ΔyieH</i>
3	<i>ΔybhA</i>	18	<i>ΔlpxM</i>	33	<i>ΔyfbT</i>	48	<i>ΔyigB</i>
4	<i>ΔybiV</i>	19	<i>ΔyedL</i>	34	<i>ΔyphH</i>	49	<i>ΔyigL</i>
5	<i>ΔycaC</i>	20	<i>Δddg</i>	35	<i>ΔyphI</i>	50	<i>ΔysgA</i>
6	<i>ΔrarA</i>	21	<i>ΔcaiE</i>	36	<i>ΔygaP</i>	51	<i>ΔeptA</i>
7	<i>ΔymdC</i>	22	<i>ΔwbbJ</i>	37	<i>ΔyqaB</i>	52	<i>ΔyifP</i>
8	<i>ΔyefH</i>	23	<i>ΔwcaF</i>	38	<i>ΔyghX</i>	53	<i>ΔcheB</i>
9	<i>ΔycgM</i>	24	<i>ΔwcaB</i>	39	<i>ΔyghY</i>	54	<i>ΔyieG</i>
10	<i>ΔyciA</i>	25	<i>ΔfrmB</i>	40	<i>ΔygpP</i>	55	<i>ΔyqiA</i>
11	<i>ΔycjT</i>	26	<i>ΔtesB</i>	41	<i>ΔuxaA</i>	56	<i>ΔbioH</i>
12	<i>ΔycjY</i>	27	<i>Δaes</i>	42	<i>ΔyheT</i>	57	<i>ΔyijU</i>
13	<i>ΔynbC</i>	28	<i>ΔtesA</i>	43	<i>Δphp</i>	58	BW25113
14	<i>ΔyniC</i>	29	<i>ΔybgC</i>	44	<i>ΔyrfG</i>	59	BL21(DE3)
15	<i>ΔyecD</i>	30	<i>ΔybhC</i>	45	<i>ΔeptB</i>	60	BL21/ pAW31

The numbers correspond to the TLC lanes shown in Fig. 2A. BioH was shown to be the sole enzyme responsible for DMB-S-MMP hydrolysis.

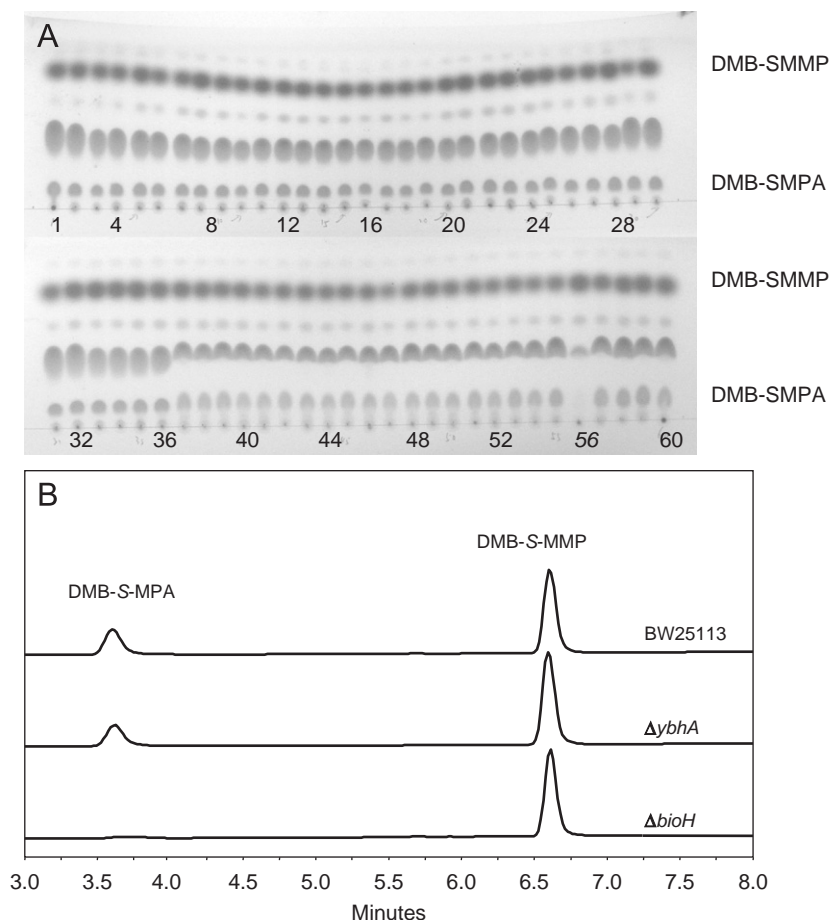


Fig. 2. (A) Thin layer chromatography showing the organic extracts of 60 *E. coli* strains supplemented with DMB-S-MMP. The strains identifications can be found in Table 1. The TLC plate was developed with 20% EA in hexane to separate DMB-S-MMP (top) and DMB-S-MPA (bottom). The middle spot is an unknown compound. In all strains except #56, DMB-S-MMP was hydrolyzed to DMB-S-MPA after 10 h of incubation. The  $\Delta bioH$  strain did not hydrolyze DMB-S-MMP. (B) Confirmation of TLC results using HPLC. No DMB-S-MPA can be detected in the extract of  $\Delta bioH$  strain. In contrast, deletion of a different putative esterase gene *ybhA* had no effect on the hydrolysis reaction. The spectra were collected at 234 nm.

extent of hydrolysis was measured by HPLC (234 nm). BioH exhibited Michaelis–Menten kinetics towards DMB-S-MMP and the  $k_{cat}$  and  $K_m$  values were determined to be  $260 \pm 45 \text{ s}^{-1}$  and  $229 \pm 26 \mu\text{M}$ , respectively (Fig. 3).

The activities of BioH towards other dimethylbutyryl thioesters were also examined using the HPLC assay. Under identical reaction conditions (1 mM substrate, 10 nM BioH), we observed that BioH displayed most the potent esterase activity towards DMB-S-MMP ( $V = 21.5 \text{ mM/s}$ ). Decreasing the carboxylic acid backbone length by one carbon (DMB-S-MTG) led to a 2.2-fold decrease in the rate of hydrolysis ( $V = 9.6 \text{ mM/s}$ ), while increasing the size of the ester moiety to an ethyl ester (DMB-S-EMP) significantly attenuated the rate of substrate hydrolysis ( $V = 1.7 \text{ mM/s}$ ). These observations are consistent with the *in vivo* result, where these substrates were each hydrolyzed in the presence of cells, albeit to lesser extents than DMB-S-MMP.

BioH is an essential enzyme in the biosynthesis of biotin in *E. coli* (Lemoine et al., 1996). It has been proposed that BioH is responsible for synthesizing pimeloyl-CoA (Guillen-Navarro et al., 2005), but its exact biochemical function

has not been confirmed. Interestingly, the crystal structure of *E. coli* BioH has been determined to 1.7 Å resolution in an effort to predict protein function from structural features (Sanishvili et al., 2003). High-throughput structural analysis unveiled a catalytic triad in BioH that is also found in known hydrolases, which hinted BioH may possess hydrolytic activity. Assays using *p*-nitrophenol esters showed that BioH displays carboxylesterase activities with preference towards short chain fatty acid esters (Sanishvili et al., 2003). Our work, both *in vivo* and *in vitro*, further elaborates the biochemical properties of BioH and shows that the enzyme has very broad substrate specificity towards esters moieties. In contrast, BioH displayed no thioesterase activities towards the thioester bond present in the substrates analyzed in this work. No further degradation of the hydrolyzed thioester acids were observed.

### 3.3. Construction of BL21(DE3) $\Delta bioH$ mutant YT2

After identification and verification of BioH as the enzyme responsible for hydrolyzing DMB-S-MMP during

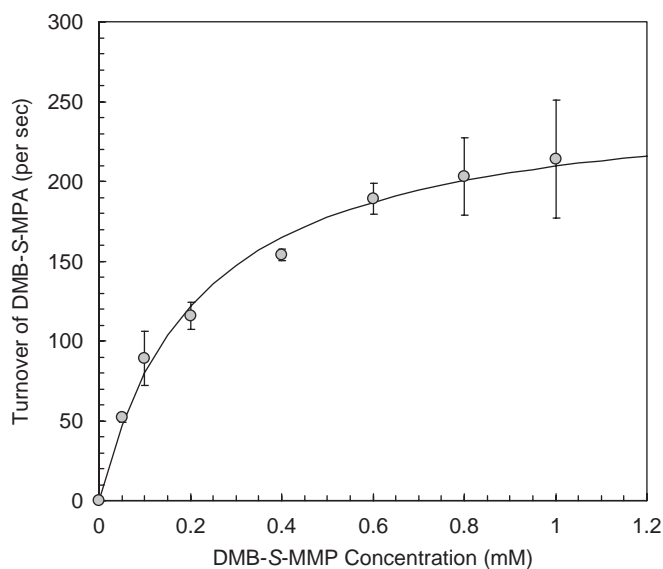


Fig. 3. Michaelis–Menten kinetics of BioH towards DMB-S-MMP. The results are averaged for three runs. The  $k_{cat}$  and  $K_m$  values are  $260 \pm 45 \text{ s}^{-1}$  and  $229 \pm 26 \mu\text{M}$ , respectively. The high standard deviation observed at high DMB-S-MMP concentration may be due to the poor solubility of the substrate in buffer (50 mM HEPES, pH 7.9, 10% DMSO).

fermentation, it was evident that a BioH deficient *E. coli* strain should be used as the host for whole-cell biosynthesis of simvastatin acid. We constructed various expression vectors of LovD that do not require the T7 polymerase and transformed them into JW3357. Evaluation of simvastatin acid bioconversion rates in these strains showed the LovD activities are significantly lower than that of BL21(DE3)/pAW31 (data not shown). The slower reaction velocities are largely attributed to the lowered expression levels of LovD in these host/vector combinations, as determined by SDS-PAGE. As a result, we concluded that a  $\Delta bioH$  derivative of BL21(DE3) is needed for achieving maximum conversion rates, while eliminating substrate hydrolysis.

Each of the Keio Collection single-gene knockout mutants contained a kanamycin resistance gene in place of the target gene (Baba et al., 2006). The marker is flanked by FRT sites which facilitates facile removal of the marker by the FLP enzyme. We used P1 transduction to move the  $\Delta bioH::FRT\text{-kan-FRT}$  marker from JW3357 to BL21(DE3) and yield the engineered strain YT1. The helper plasmid pCP20 (Datsenko and Wanner, 2000) was used to remove of the *kan* gene to afford the desired strain YT2 (BL21(DE3)  $\Delta bioH::FRT$ ). PCR analysis using primers annealing to the upstream and downstream regions confirmed deletion of *bioH*, as well as removal of the *kan* marker (data not shown).

YT2 was then cultured in LB medium and the DMB-S-MMP hydrolysis assay was performed. As expected, the new strain catalyzed no detectable hydrolysis of the thioester and no trace of DMB-S-MPA can be found in the culture broth. DMB-S-MMP can be nearly quantitatively recovered from the saturated culture that had been

grown for >24 h, reassuring that the substrate can remain intact through prolonged fermentation using this strain.

### 3.4. Whole-cell biocatalysis using YT2

We first examined the viability of YT2 as a host for the whole-cell biocatalytic synthesis of simvastatin acid. BL21(DE3)/pAW31 and YT2/pAW31 were each grown to  $OD_{600}$  of 0.5, followed by induction of protein synthesis with  $100 \mu\text{M}$  IPTG at  $20^\circ\text{C}$  for up to 16 h. The two strains exhibited identical growth kinetics when LB media was used, reaching the same  $OD_{600}$  (3.9–4.0) at the end of protein expression period. When F1 minimal medium was used, the two strains grew comparably before induction. In contrast, YT2/pAW31 grew considerably slower than BL21(DE3)/pAW31 in medium without biotin supplementation after induction. The post-induction cell density for the mutant strain ( $OD_{600} \sim 2.5$ ) was  $\sim 60\%$  of the parent strain ( $OD_{600} \sim 4.1$ ). We attributed the retarded growth rate to the inability of YT2/pAW31 to synthesize biotin and support robust cell growth in the minimal medium. When YT2/pAW31 strain was grown in F1 medium supplemented with  $0.15 \text{ mg/l}$  biotin, the growth kinetics of the mutant strain were indistinguishable from that of BL21(DE3)/pAW31.

We then compared the efficiency of YT2/pAW31 to BL21(DE3)/pAW31 in the whole-cell assay. Both strains were grown in LB medium and were concentrated 10-fold to mimic a high cell density environment after 12 h of LovD expression. MJ acid and DMB-S-MMP were added to final concentrations of 15 and 25 mM, respectively, to initiate the bioconversion. Fig. 4 shows that YT2/pAW31 was significantly more efficient in the assay as a result of *bioH* deletion. Complete conversion (>99%) was observed for the mutant strain in less than 12 h of incubation with no trace of DMB-S-MMP hydrolysis. In contrast, BL21(DE3)/pAW31 achieved the same conversion in 24 h, while requiring addition of another 25 mM of DMB-S-MMP during the fermentation as a result of substrate hydrolysis. During the linear range of the reaction, YT2/pAW31 synthesized simvastatin acid at a rate of  $1.5 \text{ mM/h}$ , significantly higher than the  $0.75 \text{ mM/h}$  measured for BL21(DE3)/pAW31 (Xie and Tang, 2007). Using YT2/pAW31, we were also able to decrease the minimal concentration of DMB-S-MMP required to drive the reaction to completion. Complete conversion to simvastatin acid can be achieved with identical rates when the initial DMB-S-MMP (18 mM) to MJ acid (15 mM) molar ratio was as low as 1.2.

The enhancement in rate is presumably due to the increased intracellular concentration of DMB-S-MMP in the absence of BioH. From the *in vitro* studies, we showed that BioH is extremely rapid in hydrolyzing DMB-S-MMP. Therefore, the competing hydrolysis reaction can rapidly deplete the pool of substrates that diffuse into the cytoplasm. Maintaining elevated intracellular concentration of DMB-S-MMP is critical, especially considering

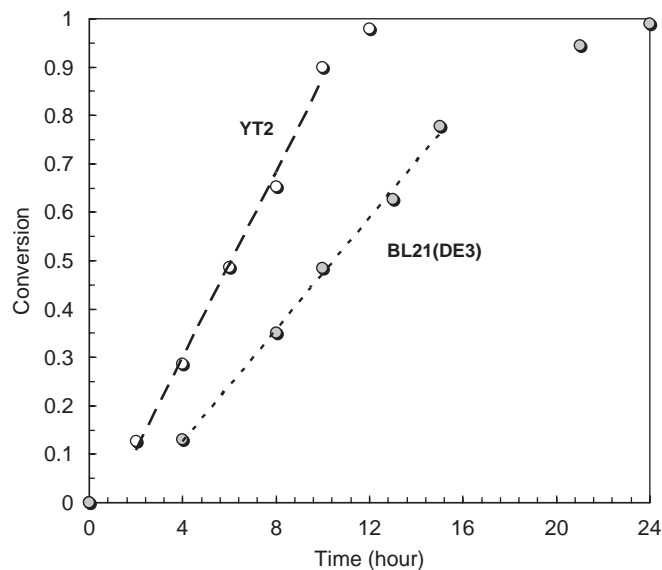


Fig. 4. Comparison of simvastatin conversion as a function of time (15 mM MJ, 25 mM DMB-S-MMP). YT2/pAW31 (○) is significantly faster than BL21(DE3)/pAW31 (●) (data taken from Xie and Tang, 2007) in reaching 99% conversion. Both strains displayed a lag phase immediately following substrate addition (0 h) and a lag phase near reaction completion. The reaction velocities between the lag phases are linear. The conversion rate for YT2/pAW31 (1.5 mM/h) is twice as fast as that for BL21(DE3) (0.75 mM/h).

LovD can catalyze the reversible reaction in which simvastatin acid can be hydrolyzed to MJ acid in the absence of acyl thioester donors (Xie et al., 2006).

Purification of simvastatin acid after bioconversion was also improved as demonstrated by working up a scaled-up fermentation of YT2/pAW31 (200 ml, 15 mM MJ acid, 18 mM DMB-S-MMP). After verification of complete conversion of MJ acid into simvastatin acid by HPLC (Fig. 5, trace a), the combined fermentation broth and cell extract was washed with hexane, acidified with 6 M HCl and filtered to collect the precipitated simvastatin acid (trace b). After washing the filter cake with 1 volume of dH<sub>2</sub>O, simvastatin acid was solubilized in ACN, filtered and analyzed by HPLC (trace c). No additional washing steps were required to remove DMB-S-MPA that was present in BL21(DE3)/pAW31. The final recovery of simvastatin acid using this approach was 94%.

#### 4. Conclusion

We have used Keio single-gene knockout library to identify BioH as the carboxylesterase that hydrolyzes DMB-S-MMP during whole-cell biocatalytic conversion of simvastatin acid from MJ acid. BioH exhibits very rapid ester hydrolysis rates, which depletes the intracellular concentration of DMB-S-MMP available as an acyl donor in the LovD-catalyzed transesterification. Using the  $\Delta bioH$  expression strain YT2, we were able to completely eliminate degradation of DMB-S-MMP and significantly increase the robustness of the whole-cell biocatalyst. This

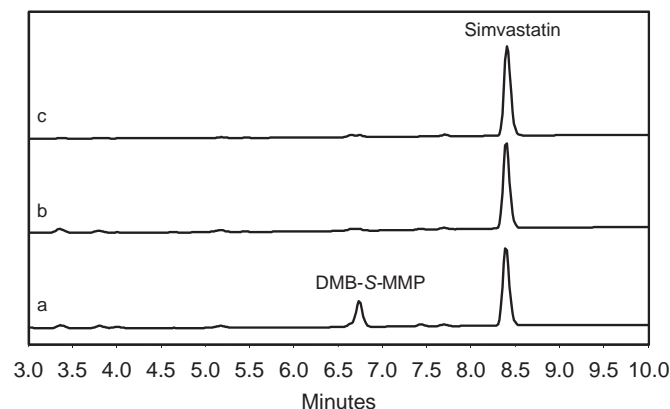


Fig. 5. HPLC analysis (238 nm) of simvastatin purification steps. Simvastatin acid is lactonized to simvastatin prior to injection. Trace a: crude extract (combined culture broth and cell extract) of YT2/pAW31 after completion of reaction (99% MJ acid conversion to simvastatin acid). The peak at 6.80 min is DMB-S-MMP; Trace b: crude sample after washing with equal volume of hexane to remove unreacted DMB-S-MMP and precipitation; Trace c: purified simvastatin after washing with dH<sub>2</sub>O and solubilization in ACN. The small shoulder peak seen at 6.7 min is simvastatin acid that has not been completely lactonized (~1%).

strain may be a useful host in other precursor directed biosynthesis and biocatalysis applications where one or more substrates used contains a labile ester linkage (Murli et al., 2005).

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