ACS Catalysis

Research Article

pubs.acs.org/acscatalysis

Directed Multistep Biocatalysis for the Synthesis of the Polyketide Oxytetracycline in Permeabilized Cells of *Escherichia coli*

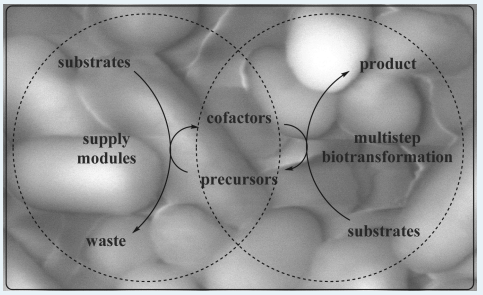
Steffen Krauser,[†] Thomas Hoffmann,[‡] and Elmar Heinzle*[,†]

[†]Biochemical Engineering, Saarland University, Campus A1.5, 66123 Saarbrücken, Germany

[‡]Helmholtz Institute for Pharmaceutical Research Saarland, Helmholtz Centre for Infection Research and Department of Pharmaceutical Biotechnology, Saarland University, Campus C2.3, 66123 Saarbrücken, Germany

Supporting Information

ABSTRACT: We report on tailored regeneration modules for the essential cofactors ATP and NADPH as well as for SAM supply, adapted to support biocatalytic multistep synthesis of oxytetracycline (OTC) in permeabilized cells of *E. coli*. ATP is regenerated from ADP and AMP by the use of polyphosphate. The efficiency of this system was proven with permeabilized cells producing flaviolin. NADPH is regenerated from $NADP^+$ by oxidation of phosphonate. A constant level of 0.7 mg/L of SAM is provided by the ligation of ATP and methionine. OTC is synthesized at a rate of 2.05 ng/(h (g of the combined biocatalyst)), reaching a final concentration of 0.9 mg/L.



KEYWORDS: *biocatalysis, Escherichia coli, permeabilized cells, adenosine-5′-triphosphate, β-nicotinamide-adenine-dinucleotide-phosphate, S-adenosylmethionine, oxytetracycline, flaviolin*

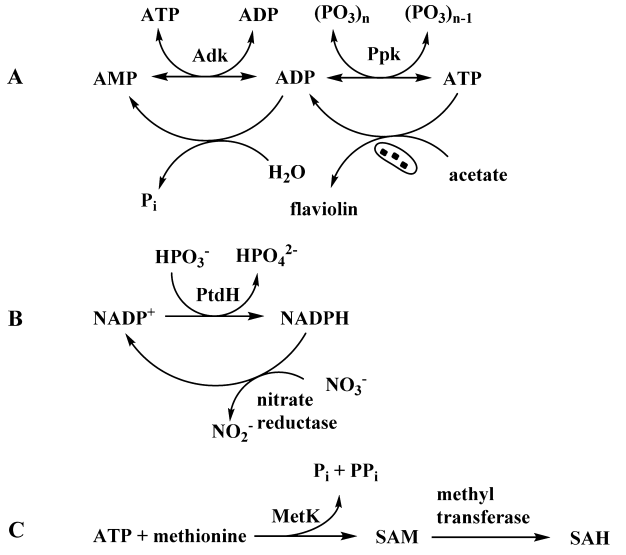
INTRODUCTION

Secondary metabolites are powerful leading structures for the development of highly active pharmaceuticals. Nature's potential to convert simple substrates selectively to complex compounds of high value has been unreached by organic chemistry.[1] Most secondary metabolites are synthesized in enzyme clusters made up of several unique subunits carrying out sequential tailoring of the carbon backbone. Megasynthases channel the respective substrate toward the next catalytic active site, thus reaching the highest selectivity. Directed multistep biocatalytic reactions in permeabilized cells, so-called in situ biocatalysis, is a promising method to close the gap between common in vitro and in vivo synthesis strategies.[2] Mild permeabilization of the cell wall enables access to megasynthases under native conditions.[2] Using permeabilized cells offers applications using megasynthases for the selective synthesis of complex carbon structures, e.g. oxytetracycline (OTC), which is synthesized by a polyketide type II megasynthase coded by the *oxy* gene cluster of *Streptomyces rimosus*. The biosynthesis of OTC was recently elucidated by Pickens et al.[3] Further, the OTC megasynthase was functionally expressed in *E. coli* by Boddy and Garza via heterologous expression of the OTC gene cluster with coexpression of the σ^{54} factor of *Myxococcus xanthus*.[8] We previously reported on the synthesis of the polyketide secondary metabolite flaviolin in permeabilized *Escherichia coli* cells using a polyketide type III synthase.[5] With acetate as the starting material, it was possible to synthesize significant amounts of flaviolin consuming only acetate and ATP. Catalytic amounts of Coenzyme A were efficiently employed to provide malonyl-CoA for polyketide synthesis. Therefore, biocatalytic in situ synthesis of OTC should be carried out using tailored cells of *E. coli* that are supported by a number of modular cofactor supply biocatalysts (Scheme 1). However, there is a high demand for cofactors, as they are usually washed out during the preparation of permeabilized cells. Cofactor regeneration is a key task in many biocatalytic conversions, particularly for multistep biocatalysis.[3,4,9] The efficiency of enzymatic reactions demanding cofactors is directly correlated to the regeneration of the respective cofactor.[10] As the value of cofactors often exceeds the substrate's cost by orders of magnitude, only catalytic amounts are applicable.[11] However, industrial applications demand reliable and cheap regeneration strategies for cofactors supplying energy, redox equivalents, and monomers. We reported previously on an efficient method to provide acetyl-CoA and malonyl-CoA.[5,12] The adaptation of known in vitro regeneration methods for ATP[13] and NADPH[14,15] as well as the supply of methyl groups from SAM[16−18] are essential to carry out the effective synthesis of secondary metabolites in permeabilized cells. For in situ coupling to a consuming biocatalytic system, ATP was regenerated from ADP and AMP by kinases degrading polyphosphate[13,19−21] and can be tested by biocatalytic synthesis of flaviolin[5] (Scheme 1A). NAD(P)H supply was achieved using phosphonate dehydrogenase[14,15,22−30] supporting reductions: i.e., reducing redox indicators[14] or enzymatic reduction of nitrate[31] (Scheme 1B). SAM as a methyl donor for methyl transferases is provided by the ligation of ATP and methionine (Scheme 1C).[18,32,33] In the

Received: November 17, 2014
Revised: January 16, 2015
Published: January 20, 2015

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407−1413

Scheme 1. Supply Reactions for Essential Cofactors in Permeabilized Cells: (A) Synthesis of ATP from ADP or AMP using Polyphosphate and Polyphosphate Kinase (Ppk) or Adenosylate Kinase (Adk), Respectively; (B) Synthesis of NADPH from $NADP^+$ using Phosphonate Dehydrogenase (PtdH); (C) Synthesis of *S*-Adenosylmethionine from ATP and Methionine using *S*-Adenosylmethionine Synthase (MetK)



present work we adapted adequate cofactor supply systems for the application in permeabilized cells for the synthesis of secondary metabolites. The adapted ATP regeneration system uses adenylate kinase (Adk) and polyphosphate kinase (Ppk) of *E. coli* and was successfully tested in the synthesis of the polyketide secondary metabolite flaviolin. A codon-optimized double mutant of *Pseudomonas stutzeri* phosphonate dehydrogenase (PtdH) was applied for the regeneration of NADPH. SAM was supplied via *S*-adenosylmethionine synthase (MetK) of *Corynebacterium glutamicum*. Eventually, combining these modules with polyketide type II synthase expressed in *E. coli* permitted the in situ synthesis of OTC, opening up new ways for detailed studies of the megasynthase and for the production of new variants of OTC.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* BWZ3 (ΔadhE ΔackA, pta deletion mutant of *E. coli* Bl21 DE3),[12] kindly provided by the Zhao group (University of Illinois), was used as the host for the expression of vector plasmids. *E. coli* BAPI (*E. coli* Bl21 DE3 harboring genes of a chaperone set) was used for the support of correctly folded enzyme expression. Cloning vector pRSFduet-1 was obtained from Novagen. Strains and plasmids used in this study are given in Table 1. For the construction of pRSF-*adk* and pRSF-*ptdH* *E. coli* adenylate kinase (*adk*) and codon optimized E175A, A176R *P. stutzeri* phosphonate dehydrogenase double mutant (*ptdH*)[14,30] were custom synthesized by Genscript and cloned into NdeI-EcoRV and *Bam*HI-*Hin*dIII restriction sites, respectively. For the construction of pRSF-*adk*/*ppk* *E. coli* polyphosphate kinase (*ppk*) was amplified using 5′-TACCATGGGTCAGGAAAAGCTATA-3′ and 5′-ATGGATCCTTATTCAGGTTGTTCGAGTGA-3′ primers and cloned into the NcoI-*Bam*HI restriction site of pRSF-adk.[13] S-adenosylmethionine synthase (*metK*) of C. *glutamicum* with start codon change to ATG was amplified using 5′-TGAGCTCATGGCTCAGCCAACCGCC-3′ and 5′-TAAGCTTTTAGGCCAACTTGAGGGCTGCG-3′ primers and cloned into the SacI-*Hin*dIII restriction site of pRSFduet-1.[33] *E. coli* BWZ3 was transformed with vector plasmids using standard methods. Strains containing plasmids were cultured in LB media supplemented with 20 mM $MgSO_4$ and 50 μg/mL of kanamycin. Baffled flasks were shaken at 230 rpm and 37 °C. Expression of vector genes was induced by addition of 1 mM IPTG to the culture followed by 2 h of incubation at 230 rpm and 28 °C. Protein expression was checked after disruption of cells with glass beads using a standard trypsinization protocol with subsequent MALDI-TOF MS peptide mass fingerprint analysis.[36]

Permeabilization of *E. coli* BWZ3. Cells were harvested at an OD_{660} value of 1.8 by centrifugation and washed with Tris buffer. The cell density was set to 50 mg/mL cell wet weight, and 0.25% (w/v) Triton X-100 was added to the suspension from a 5% (w/v) stock solution. The permeabilization was carried out for 15 min at 30 °C with shaking in Falcon tubes at 230 rpm. Permeabilized cells were washed with Tris buffer (0.1 M) three times and resuspended in a suitable reaction buffer.[5] The permeabilization efficiency was checked with a glucose-6-phosphate activity assay, as in previous studies.[5]

Enzyme/Redox Reactions. Cells were resuspended in 0.1 M Tris HCl/KCl buffer, pH 7.8, 0.05 mg/mL phenazine methosulfate (PMS), and 0.1 mg/mL nitro blue tetrazolium (NBT) or indophenol blue (DCPIP), respectively.[14,37] The redox reaction was started with the addition of $NaHPO_3$ and $NADP^+$ from stock solution. Regeneration of ATP was

Table 1. Strains and Plasmids

name	information	source
E. coli BWZ_3	*E. coli* BL21 DE3 Δ *ackA*/*pta* Δ *adhE*	Zhao, University of Illinois[12]
E. coli BAPI	*E. coli* BL21 DE3 with a set of chaperones	Boddy, University of Ottawa[34]
pRSF-*adk*/*ppk*	pRSFDuet-1 carrying sequences of adenylate kinase (*adk*) and polyphosphate kinase (*ppk*) of *E. coli*	this study
pRSF-*metK*	pRSFDuet-1 carrying *S*-adenosylmethionine synthase sequence (*metK*) of *C. glutamicum*	this study
pRSF-*ptdH*	pRSFDuet-1 carrying E175A/A176R codon optimized double mutant sequence of *P. stutzeri* phosphonate dehydrogenase (*ptdH*)	this study, Genscript
pACYC-*acs*	pACYCDuet carrying acetyl-CoA synthase (*acs*) sequence of *E. coli*	Zhao, University of Illinois[12]
pET-*rppA*	pET-16b vector carrying rppA polyketide type III synthase sequence of *Streptomyces griseus*	Ohnishi, Tokyo University[35]
pMRH08	carrying OTC gene cluster of *S. rimosus* and *asgE* promoter region of *M. Xanthus*	Boddy, University of Ottawa[34]
pDSC02	carrying σ^{54}-factor (*rpoN*) of *M. Xanthus*	Boddy, University of Ottawa[34]

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407–1413

observed by a flaviolin synthesis assay, as previously described.[5] NADPH-dependent reduction of nitrate via nitrate reductase from Boehringer and subsequent quantification of diazo-dye formation was performed using the standard Boehringer NO_3^-/NO_2^- protocol,[31] on which phosphonate did not show any effect. Colorimetric detection was performed using a helios α photometer.

Separation, Detection, and Quantification of SAM. All measurements of SAM were performed on a Dionex Ultimate 3000 RSLC system using a Waters BEH C18, 50 × 2.1 mm, 1.7 μm dp column by injection of a 1 μL sample.[38] Separation was achieved by isocratic elution with H_2O + 0.1% formic acid at a flow rate of 350 μL/min and 45 °C. Coupling the HPLC to an MS was supported by an Advion Triversa Nanomate nano-ESI system attached to a Thermo Fisher Orbitrap. SAM was quantified using a single reaction monitoring method performed in the linear ion trap. The parent ion was isolated at m/z 399.1 ± 1 and fragmented by CID at an energy of 35 eV. SRM-based peak areas of the characteristic m/z 250 fragment corresponding to the loss of methionine were used for quantification. A five-point calibration curve was generated from triplicate measurements. Full-scan mass spectra were acquired in parallel ranging from m/z 150 to 500 at a resolution of R = 30000.

Separation, Detection, and Quantification of OTC. UPLC analysis was carried out on an Agilent Infinity UPLC system using a Gemini 5U C_{18} 110A, 150 × 4.6 mm column. Separation of 20 μL sample volume was achieved using acetonitrile/0.1% phosphoric acid applying gradient elution from 7.5% to 25% acetonitrile over 9.5 min at a flow rate of 0.8 mL/min. Detection of OTC occurred using the diode array detector (DAD) at 365 nm. Peak areas of the elution peaks at 7.054 and 7.493 min were used for quantification. A nine-point calibration curve was generated from triplicate measurements. For MALDI-TOF MS analysis, samples were mixed 1:1 with saturated 9-aminoacridine in MeOH and analyzed in the negative reflector mode at laser intensities between 3700 and 4600 with an Applied Biosystems 4800 MALDI-TOF/TOF analyzer. High-resolution MS data were generated using MALDI-FTICR-MS analysis. Samples of MALDI-TOF analysis were transferred to a MALDI target for analysis in a Bruker solariX ESI/MALDI-FTICR-MS. The MALDI source with pulsed ND:YAG laser was operated at −100 V plate offset and −180 V deflector plate with 50% laser intensity. Ions were directly desorbed into an octopole ion guide (5 MHz) while being cooled by liquid helium. Ions generated by 250 laser shots were accumulated in the octopole at 14 V and extracted at 7 V into the analyzer cell.

Synthesis of OTC. The following mixture was prepared for synthesis with OTC megasynthase: permeabilized cells of five strains, each 25 g/L (*E. coli* BAPI pMRH08/pDCS02, *E. coli* BWZ3 pRSF-*adk/ppk*, *E. coli* BWZ3 pRSF-*ptdH*, *E. coli* BWZ3 pRSF-*metK*, and *E. coli* BWZ3 pRSF-*acs* (Table 1). Permeabilized cells were resuspended in reaction buffer (100 μM ATP, 50 μM $NADP^+$, 50 μM CoA, 50 μM methionine, 40 mM NH_4OAC, 4 g/L sodium phosphonate, and 4 g/L sodium polyphosphate in 0.1 M NH_4HCO_3 buffer at pH 7.8). Samples of 1 mL were centrifuged at 8500*g*, and the supernatant was subjected to analysis.

RESULTS AND DISCUSSION

NADPH Supply. As reported by Woodyer et al., the E175A, A176R double mutant of *P. stutzeri* phosphonate dehydrogenase (ptdH) shows a significant increase in $NADP^+$ acceptance.[14] For reasons of enhanced protein expression, codon optimization for *E. coli* was performed. Cells of permeabilized *E. coli* BWZ3 carrying the pRSF-ptdH vector were able to generate NADPH. In redox assays with $NADP^+$ and permeabilized cells of *E. coli* BWZ 3, the NADPH-selective redox indicators NBT (0.1 mM) and DCPIP (1 mM) were completely converted into their respective reduced states. Coupled to NADPH-consuming enzymatic reduction of nitrate via nitrate reductase, permeabilized cells, as well as native cells of *E. coli*, showed NADPH generation activity. For the reduction of nitrate via nitrate reductase, NADPH is essential and generates equimolar amounts of nitrite. Phosphonate did not interfere with this assay in any way. Due to substrate inhibition, nitrate levels were kept below 10 mM.[39] Despite several combinatorial reaction setups, the overall amount of nitrite produced did not exceed 0.1 mM (Figure 1). Further

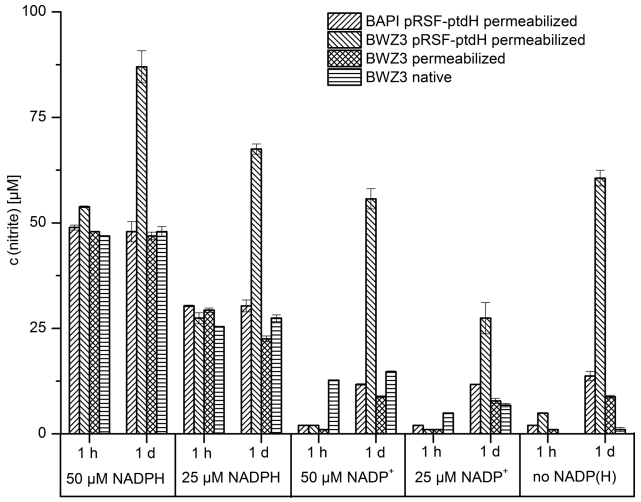


Figure 1. Nitrite production by NADPH-consuming reduction of nitrate via nitrate reductase. Reactions were carried out in Tris-HCl buffer (0.1 M) containing 4 g/L sodium phosphonate, 5 mM sodium nitrate, and 50 g/L permeabilized cells of *E. coli* BAPI pRSF-*ptdH*, *E. coli* BWZ3 pRSF-*ptdH*, *E. coli* BWZ3, or native *E. coli* BWZ3 cells, respectively. Concentrations of nitrite were measured by a photometric diazo-dye assay from Boehringer[31] after 1 h and 1 day of incubation at 30 °C with shaking at 230 rpm in a Falcon tube.

investigation revealed that, unexpectedly, not only biocatalyst cells were able to provide NADPH redox equivalents from $NADP^+$ but also the NADPH-dependent enzymatic reduction was supported significantly without addition of β-nicotinamide-adenine-dinucleotide-phosphate species during 1 day incubations. We were able to measure 60 μM nitrite using 50 g/L permeabilized cells of *E. coli* BWZ3 pRSF-*ptdH* without addition of β-nicotinamide-adenin-dinucleotide species. Probably enzyme-bound NADPH moieties provide the needed substrate. This effect only appeared when cells were permeabilized prior to their use in the reaction and was lower without overexpression. Sequence analysis of pRSF-*ptdH* indicated correct insertion of the *P. stutzeri* PtdH E175A, A176R double mutant into the *Bam*HI-*Hin*dIII restriction site. The *Bam*HI site of the pRSFduet-1 vector provides a His-Tag which was used to purify the expressed enzyme with Dynabeads. A protein expression check revealed that the expression level of *P. stutzeri* ptdH in *E. coli* BWZ 3, as well as in *E. coli* BAPI, was low. However, the use of Dynabeads,

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407−1413

loaded with the protein, slightly increased the amount of nitrite produced by the enzymatic reduction of nitrate. Since pRSFduet-1 protein expression levels for heterologous genes are usually high in *E. coli* BL21 DE3, we assume that created RNA or protein is quickly degraded by the expression host. The best expression conditions were found at 2.5 h of incubation with 1 mM IPTG at 28 °C characterized by the activity of generation of NADPH from $NADP^+$ by enzymes pulled down using Dynabeads. Also, native cells were able to convert $NADP^+$ to NADPH, which indicates the potential for extracellular reduction, as previously observed by others.[40] O-Overexpression of *ptdH* was apparently limited by cellular mechanisms: i.e. degradation of m-RNA or protein as well as regulatory influence on the vector itself. This has to be investigated in more detail. Though the regeneration rate of NADPH was not very high, it seems sufficient for secondary metabolite production.

ATP Supply. ATP regeneration is achieved by phosphorylation of ADP via polyphosphate kinase (Ppk).[21,41−45] Polyphosphate is a native energy storage material within cells and is accumulated during growth on energy-rich media.[46−48] Phosphorylation of NDPs in the presence of polyphosphate is reversible, thus reaching equilibrium. It has been shown that the kination efficiency for ADP can reach 52.3%.[13] In our study, polyphosphate kinase was overexpressed in cells of *E. coli* BWZ3. Cells with overexpressed *ppk* were permeabilized and used as an ATP regeneration module in the biocatalytic synthesis of flaviolin. The yield of flaviolin was correlated to ATP consumption where the synthesis of one molecule of flaviolin consumes five molecules of ATP.[5] This resulted in up to six regeneration cycles for ATP. Interestingly, the amount of flaviolin detected in reactions without addition of polyphosphate indicates regeneration as well. We assume this is caused by enzyme-bound polyphosphate synthesized during the growth of catalyst cells as the overexpression of *ppk* triggers accumulation of polyphosphate.[20,47,49] Further MALDI-TOF analysis of the reaction supernatant revealed that ATP was degraded to AMP during the reaction. This is thought to be caused by hydrolysis of ADP. We were able to enhance the regeneration efficiency by coexpression of *E. coli* adenosylate kinase (Adk), which uses ATP to phosphorylate AMP. Flaviolin concentrations of 130.3 μM reached with permeabilized cells overexpressing *adk*/*ppk* indicate a production of 1.3 mmol of ATP per liter. The catalytic amount of ADP (50 μM) was therefore recycled at least 26 times. With regard to the initial yields of only 30% for ATP, without expression of adk/ppk (Table 2), the biocatalytic efficiency was increased 90-fold using the *E. coli* ATP regeneration module.

SAM Supply. *S*-Adenosylmethionine is essential for methylation of carbon backbones in secondary metabolite synthesis.[50] The molecule itself is fairly unstable at physiological pH. Unlike the aforementioned molecules, SAM decomposes to homocysteine and adenosine after transfer of the methyl moiety. In this case regeneration would be very difficult if not impossible in an in situ biocatalytic system. As the *S*-adenosylmethionine synthase (MetK) is strongly feedback inhibited,[18] stock synthesis is also not favorable. However, for biocatalytic reactions a constant level of SAM would be sufficient to provide the methyl donor. Reactions consuming ATP also would benefit from synthesis on demand. In order to achieve this, *C. glutamicum* S-adenosylmethionine synthase (*metK*) is overexpressed in *E. coli* BWZ3, resulting in an efficient SAM supply in permeabilized cells.[33] A concentration of 0.7 mg/L SAM was reached for 16 h, while the initial activity of the catalyst was 1.7 μg/(min (g of catalyst)) (Figure 2).

Table 2. ATP Consumption Observed in a Biocatalytic in Situ Synthesis Reaction Producing Flaviolin (10 Molecules of ATP/Flaviolin) in the Presence of Acetate and Catalytic Amounts of Coenzyme A (50 μM)[5]

mixture of permeabilized cells of *E. coli* BWZ3 pRSF-*ppk* (50 mg/mL) and *E. coli* BWZ3 pACYC-*acs*/pET-*rppA* (50 mg/mL) incubated for 16 h with	$C_{flaviolin}$ (μM)	ATP turnover
ATP 30 mM (without expression of *ppk*)[5]	920	0.30
ATP 50 μM	22.2	4.45
ATP 50 μM + polyphosphate glass 10 mM	33.7	6.75
ATP 50 μM + meta-phosphate 10 mM	34.5	6.90
no ATP	0.0	
reaction buffer (reference)	0.0	
mixture of permeabilized cells of *E. coli* BWZ3 pRSF-*ppk*/*adk* (50 mg/mL) and *E. coli* BWZ3 pACYC-*acs*/pET-*rppA* (50 mg/mL) incubated for 16 h with	**$C_{flaviolin}$ (μM)**	**ATP turnover**
ADP 50 μM + polyphosphate 10 mM	130.3	26.05
no ADP	0.0	

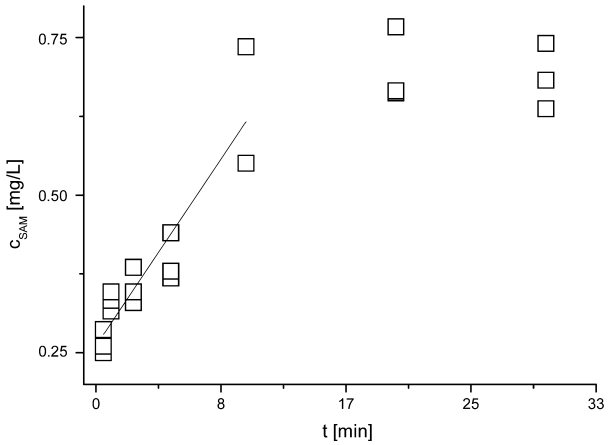
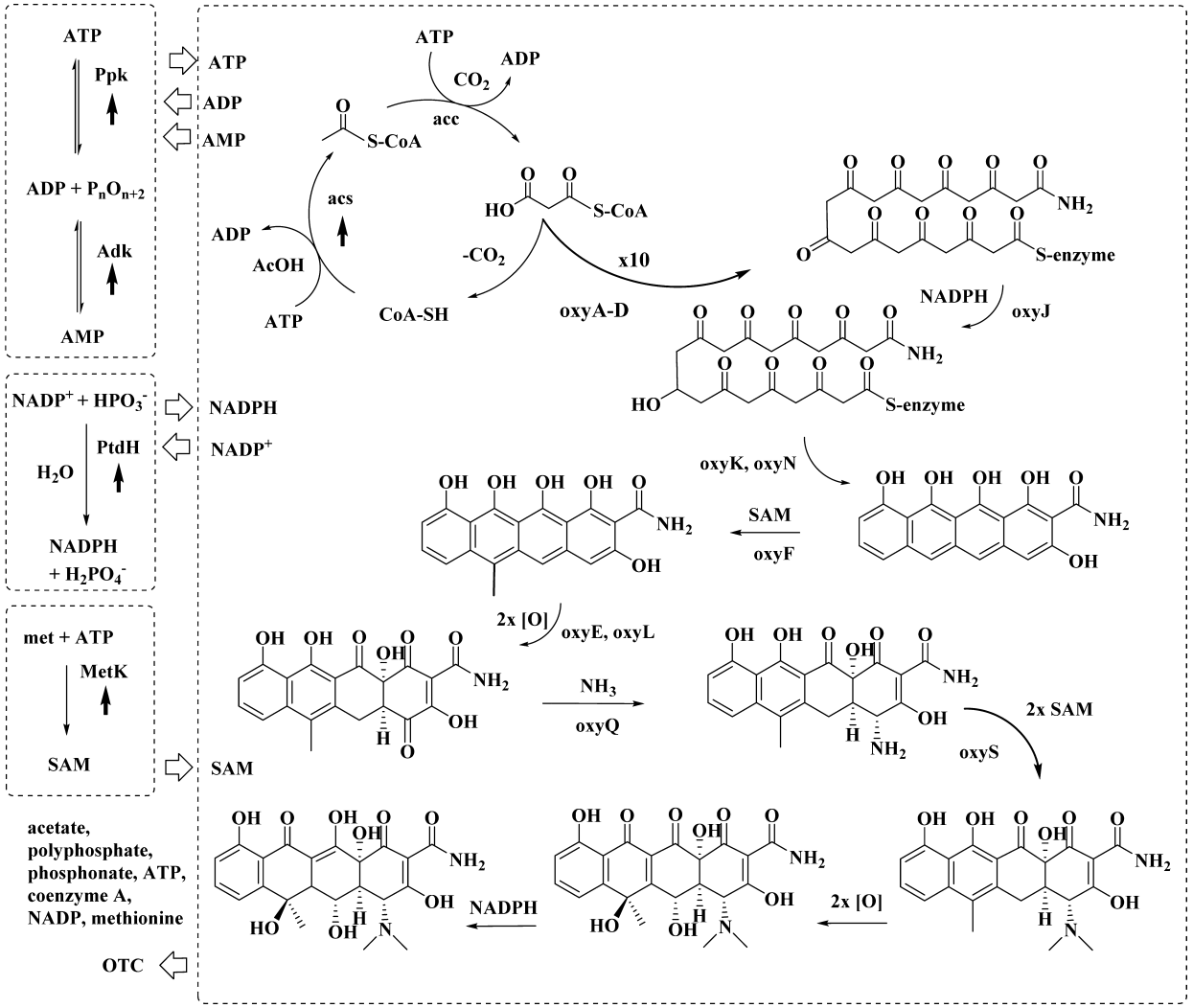


Figure 2. Time course of SAM concentration in 0.1 M ammonium acetate reaction buffer containing 10 mM ATP, 10 mM methionine, and 50 mg/mL permeabilized *E. coli* BWZ3 pRSF-*metK*.

Synthesis of OTC. The synthesis of oxytetracycline is carried out by nine successive elongation steps using malonyl-CoA for the construction of the polyketide backbone. The malonamate starting unit, resulting in the C2 amide, is synthesized from malonyl-CoA by oxyD. Further, cyclization, keto reduction, hydroxylation, and methylation steps tailor the backbone toward OTC (Scheme 2). The OTC megasynthase was functionally expressed in *E. coli*.[34] We were able to successfully use the enzyme cluster in permeabilized cells of *E. coli* BAPI when the synthesis was supported by previously developed supply modules for ATP, NADPH, and SAM. When an in situ supply of cofactors was used via the corresponding synthesis modules, oxytetracycline (*m*/*z* 459.14) was synthesized. The reaction mixture showed initial OTC formation (Figure 3). However, OTC was further converted in permeabilized *E. coli*, resulting in the accumulation of dihydrooxytetracycline (H_2OTC, *m*/*z* 461.15), presumably obtained by the reduction of one of the keto functional groups. The chemical formula of H_2OTC was confirmed by high-resolution MS data with only 1.25 ppm deviation from the theoretical *m*/*z* value (Supporting Information). Without a

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407−1413

Scheme 2. In Situ Synthesis of Oxytetracycline[a]



[a]Dashed boxes indicate permeabilized cells.

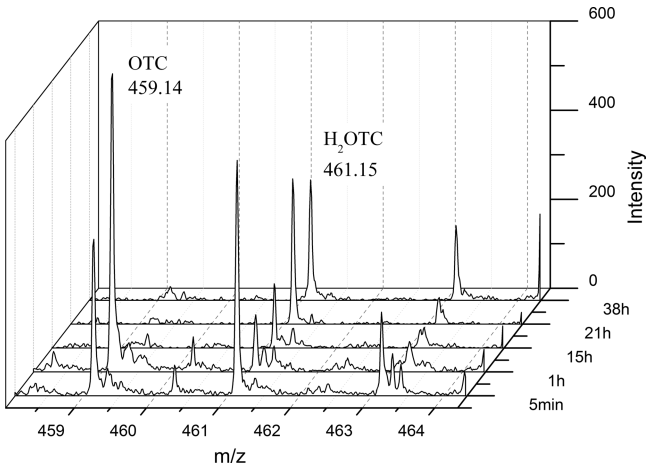


Figure 3. MALDI-TOF MS analysis of in situ OTC synthesis. Mass spectra were generated from samples of the reaction mixture supernatant at 5 min, 1 h, 15 h, 21 h, and 38 h applying the negative reflector mode. Samples were cocrystallized 1/1 (v/v) with the 9-aminoacridine matrix (9 mg in MeOH).

biocatalytic cofactor supply, OTC synthesis could not be observed. Synthesis of OTC in permeabilized cells of *E. coli* BAPI carrying pMRH08 and pDCS02 expression vectors by external supply of malonyl-CoA, SAM, NADPH, and ATP did not succeed either. OTC (t_R = 7.054 min) and H_2OTC (t_R = 7.493 min) were both quantified as reaction products (Figure 4). The productivity of the 1 g biocatalyst mixture could be determined as 2.05 ng/h, reaching a final concentration of 0.93 mg/(L of OTC/H_2OTC). OTC synthesis in permeabilized cells is followed by reductive degradation, resulting in H_2OTC as the major product. Further degradation of the single reduced OTC cannot be excluded. In case of further degradation, the actual production rate would be higher than that determined.

CONCLUSION

Our results show that in situ synthesis of complex secondary metabolites using megasynthase clusters can be achieved using directed multistep biocatalysis. Design of viable host organisms with an adapted metabolic network is mandatory. In the case of polyketide secondary metabolites, starting from acetate is highly beneficial and results in no significant byproducts.[5] Tailoring the host organism for avoiding reductive degradation of OTC might significantly improve the synthesis. Deletion of

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407−1413

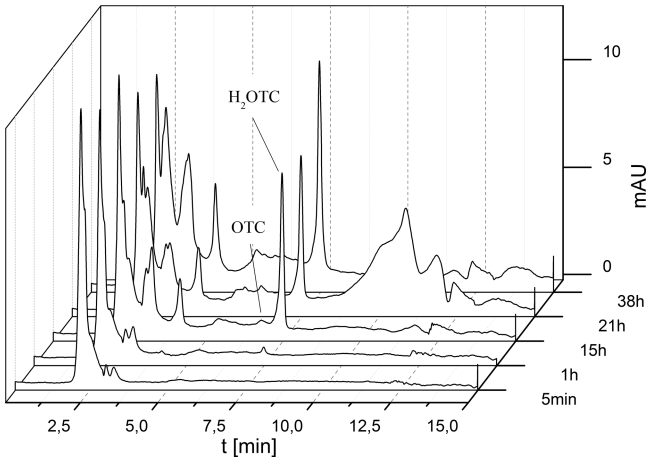


Figure 4. UPLC analysis of OTC in situ synthesis. Chromatograms were obtained from 20 μL of reaction mixture supernatant at 5 min, 1 h, 15 h, 21 h, and 38 h. OTC (t_R = 7.054 min) and H_2OTC (t_R = 7.493 min) are indicated according to the analytical standard (Sigma-Aldrich).

corresponding keto-reductase genes might lead to the primary product of the OTC synthase cluster. Since the use of permeabilized cells for biocatalytic multistep synthesis of secondary metabolites is very promising, the supply of essential cofactors is mandatory for upcoming synthesis strategies. The supply modules presented here are applicable to other strains as plasmids and can be easily transferred into strains meeting the demands of other biosynthesis routes. Significant amounts of NADPH can be provided by permeabilized cells of *E. coli*. The major application for permeabilized cell synthesis seems to be the multistep biosynthesis of secondary metabolites of high value. Especially, for the synthesis of chemically inaccessible structures and derivatives of natural substances using non-natural building blocks, in situ synthesis offers new strategies. With the economics for the given supply modules for essential biosynthetic cofactors, processes seem achievable after further strain and process optimization. In situ biosynthesis opens up new ways for mechanistic and kinetic studies of polyketide synthases to support strain development. Additionally, it offers new ways of producing chemical variants by feeding of alternative precursor molecules.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/cs501825u.

High-resolution mass analysis of dihydrooxytetracycline, kinetic data of the NADPH supply module, and synthesis of flaviolin in permeabilized cells (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail for E.H.: e.heinzle@mx.uni-saarland.de.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge Huimin Zhao, Christopher Boddy, and Anthony Garza for providing strains and plasmids. Special thanks go to Tobias Dier (Bioanalytics Institute/Saarland University) for support with FTICR-MS analysis. Further, we thank the BMBF (Federal Ministry of Education and Research, Germany) for funding the MECAT project (FKZ 031P7238 within the initiative "Biotechnologie 2020+: Basistechnologien für eine nächste Generation biotechnologischer Verfahren").

■ REFERENCES

(1) Wohlgemuth, R. *Curr. Opin. Microbiol.* **2010**, *13* (3), 283−292.
(2) Wohlgemuth, R. *J. Chem. Technol. Biotechnol.* **2007**, *82* (12), 1055−1062.
(3) Wohlgemuth, R. *Chem. Biochem. Eng. Q.* **2011**, *25* (1), 125−134.
(4) Krauser, S.; Weyler, C.; Blass, L. K.; Heinzle, E. *Fundamentals and Application of New Bioproduction Systems/Adv. Biochem. Eng./Biotechnol.* **2013**, *137*, 185−234.
(5) Krauser, S.; Kiefer, P.; Heinzle, E. *ChemCatChem* **2012**, *4* (6), 786−788.
(6) Pickens, L. B.; Tang, Y. *Metab. Eng.* **2009**, *11* (2), 69−75.
(7) Pickens, L. B.; Tang, Y. *J. Biol. Chem.* **2010**, *285* (36), 27509−27515.
(8) Stevens, D. C.; Conway, K. R.; Pearce, N.; Villegas-Penaranda, L. R.; Garza, A. G.; Boddy, C. N. *PLoS One* **2013**, *8* (5), e64858.
(9) Wohlgemuth, R. *Curr. Opin. Biotechnol.* **2010**, *21* (6), 713−724.
(10) Uppada, V.; Bhaduri, S.; Noronha, S. B. *Curr. Sci.* **2014**, *106* (7), 946−957.
(11) Ni, Y.; Holtmann, D.; Hollmann, F. *ChemCatChem.* **2014**, *6* (4), 930−943.
(12) Zha, W. J.; Rubin-Pitel, S. B.; Shao, Z. Y.; Zhao, H. M. *Metab. Eng.* **2009**, *11* (3), 192−198.
(13) Noguchi, T.; Shiba, T. *Biosci. Biotechnol. Biochem.* **1998**, *62* (8), 1594−1596.
(14) Woodyer, R.; van der Donk, W. A.; Zhao, H. M. *Biochemistry* **2003**, *42* (40), 11604−11614.
(15) Woodyer, R.; van der Donk, W. A.; Zhao, H. M. *Combinatorial Chemistry & High Throughput Screening* **2006**, *9* (4), 237−245.
(16) Aidemark, M.; Andersson, C. J.; Rasmusson, A. G.; Widell, S. *BMC Plant Biol.* 2009, *9*.
(17) Matos, J. R.; Raushel, F. M.; Wong, C. H. *Biotechnol. & Appl. Biochem.* **1987**, *9* (1), 39−52.
(18) Park, J.; Tai, J. Z.; Roessner, C. A.; Scott, A. I. *Bioorg. Med. Chem.* **1996**, *4* (12), 2179−2185.
(19) Noguchi, T.; Shiba, T. *Biosci., Biotechnol., Biochem.* **1998**, *62* (8), 1594−1596.
(20) Shiba, T.; Tsutsumi, K.; Ishige, K.; Noguchi, T. *Biochem.-Moscow* **2000**, *65* (3), 315−323.
(21) Kameda, A.; Shiba, T.; Kawazoe, Y.; Satoh, Y.; Ihara, Y.; Munekata, M.; Ishige, K.; Noguchi, T. *J. Biosci. and Bioeng.* **2001**, *91* (6), 557−563.
(22) Johannes, T. W.; Woodyer, R. D.; Zhao, H. M. *Appl. Env. Microbiol.* **2005**, *71* (10), 5728−5734.
(23) Woodyer, R. D.; Wymer, N. J.; Racine, F. M.; Khan, S. N.; Saha, B. C. *Appl. Env. Microbiol.* **2008**, *74* (10), 2967−2975.
(24) Johannes, T. W.; Woodyer, R. D.; Zhao, H. *Biotechnol. Bioeng.* **2007**, *96* (1), 18−26.
(25) Relyea, H. A.; Vrtis, J. M.; Woodyer, R.; Rimkus, S. A.; van der Donk, W. A. *Biochem.* **2005**, *44* (17), 6640−6649.
(26) Woodyer, R.; Zhao, H. M.; van der Donk, W. A. *Febs J.* **2005**, *272* (15), 3816−3827.
(27) Woodyer, R.; Chen, W.; Zhao, H. M. *J. Chem. Educ.* **2004**, *81* (1), 126−133.
(28) Woodyer, R.; Johannes, T.; van der Donk, W.; Zhao, H. M. *Abstracts of Papers*; American Chemical Society: Washington, DC, 2005; Vol. *229*, U229.
(29) Zhao, H. M.; Woodyer, R.; Johannes, T. *Abstracts of Papers*; American Chemical Society: Washington, DC, 2004; Vol. *227*, U130.
(30) Woodyer, R.; Wheatley, J. L.; Relyea, H. A.; Rimkus, S.; van der Donk, W. A. *Biochemistry* **2005**, *44* (12), 4765−4774.
(31) Beutler, H. O.; Wurst, B.; Fischer, S. *Deutsche Lebensmittel-Rundschau* **1986**, *82* (9), 283−289.

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407−1413

(32) Komoto, J.; Yamada, T.; Takata, Y.; Markham, G. D.; Takusagawa, F. *Biochemistry* **2004**, *43* (7), 1821−1831.
(33) Grossmann, K.; Herbster, K.; Mack, M. *FEMS Microbiol. Lett.* **2000**, *193* (1), 99−103.
(34) Boddy, C. N.; Garza, A., US Patent 2010, 20100184038 A1.
(35) Funa, N.; Ohnishi, Y.; Fujii, I.; Shibuya, M.; Ebizuka, Y.; Horinouchi, S. *Nature* **1999**, *400* (6747), 897−899.
(36) Hollemeyer, K.; Altmeyer, W.; Heinzle, E.; Pitra, C. *Rapid Commun. Mass Spectrom.* **2008**, *22* (18), 2751−2767.
(37) Dupuy, C.; Kaniewski, J.; Ohayon, R.; Deme, D.; Virion, A.; Pommier, J. *Anal. Biochem.* **1990**, *191* (1), 16−20.
(38) Kirsch, S. H.; Knapp, J.-P.; Geisel, J.; Herrmann, W.; Obeid, R. *J. Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2009**, *877* (30), 3865−3870.
(39) Jacques, J. G. J.; Burlat, B.; Arnoux, P.; Sabaty, M.; Guigliarelli, B.; Leger, C.; Pignol, D.; Fourmond, V. *Biochimica et biophysica acta* **2014**, *1837* (10), 1801−1809.
(40) McKinlay, J. B.; Zeikus, J. G. *Appl. Environ. Microbiol.* **2004**, *70* (6), 3467−3474.
(41) Nahalka, J.; Gemeiner, P.; Bucko, M.; Wang, P. G. *Artificial Cells Blood Substitutes and Biotechnology* **2006**, *34* (5), 515−521.
(42) Resnick, S. M.; Zehnder, A. J. B. *Appl. Env. Microbiol.* **2000**, *66* (5), 2045−2051.
(43) Nahálka, J.; Liu, Z.; Gemeiner, P.; Wang, P. G. *Biotechnol. Lett.* **2002**, *24* (11), 925−930.
(44) Kuroda, A.; Kornberg, A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94* (2), 439−442.
(45) Nocek, B.; Kochinyan, S.; Proudfoot, M.; Brown, G.; Evcokimova, E.; Osipiuk, J.; Edwards, A. M.; Savchenko, A.; Joachimiak, A.; Yakunin, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (46), 17730−17735.
(46) Meng, L.; Li, M.; Yang, S. H.; Kim, T.-J.; Suh, J.-W. *Biosci. Biotechnol. Biochem.* **2011**, *75* (8), 1576−1581.
(47) Achbergerova, L.; Nahalka, J. *Microbial Cell Factories* **2011**, 10.
(48) Achbergerova, L.; Nahalka, J. *Biologia* **2014**, *69* (3), 263−269.
(49) Rangarajan, E. S.; Nadeau, G.; Li, Y.; Wagner, J.; Hung, M.-N.; Schrag, J. D.; Cygler, M.; Matte, A. *J. Mol. Biol.* **2006**, *359* (5), 1249−1260.
(50) Loenen, W. A. M. *Biochem. Soc. Trans.* **2006**, *34*, 330−333.

DOI: 10.1021/cs501825u
ACS Catal. 2015, 5, 1407−1413