Metabolic Engineering 11 (2009) 168–177

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Establishment of a novel anabolism-based addiction system with an artificially introduced mevalonate pathway: Complete stabilization of plasmids as universal application in white biotechnology

Jens Kroll [a], Anna Steinle [a], Rudolf Reichelt [b], Christian Ewering [a], Alexander Steinbüchel [a,*]

[a] Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität, D-48149 Münster, Germany
[b] Institut für Medizinische Physik und Biophysik, Elektronenmikroskopie und Analytik, Universitätsklinikum Münster, Westfälische Wilhelms-Universität, D-48149 Münster, Germany

ARTICLE INFO

Article history:
Received 20 August 2008
Received in revised form
19 December 2008
Accepted 27 January 2009
Available online 5 February 2009

Keywords:
Addiction system
Anabolism
Mevalonate pathway
ispH
lytB
Isopentenyl pyrophosphate
IPP
Plasmid stability
Cyanophycin

ABSTRACT

Plasmid stability in recombinant microorganisms is a very important requirement for highly efficient plasmid-based production processes in biotechnology. To stably maintain plasmids, we developed in this study an efficient and stringent novel anabolism-based addiction system, which can be widely used. This novel addiction system is based on two components: (i) an *Escherichia coli* HMS174(DE3) knockout mutant of the *ispH* gene coding for 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC 1.17.1.2) of the deoxyxylulose 5-phosphate (DXP) pathway, impairing the synthesis of isopentenyl pyrophosphate (IPP) and (ii) a completely synthetic and episomal mevalonate (MVA) pathway as an alternative supplier of essential IPP. The latter is encoded by a plasmid that contains the genes for HMG-CoA reductases from *Lactococcus lactis* and *Staphylococcus aureus* plus HMG-CoA-synthase, MVA kinase, MVP kinase and MVPP decarboxylase from *S. aureus*. This plasmid should then also harbor the genes for the protein or for the pathway that will be produced or that will be utilized for production of a chemical. To demonstrate the functionality of this addiction system, a mutated cyanophycin synthetase gene ($cphA_{6308}$C595S) was used. To determine plasmid stabilities, flasks experiments in media supplied or not supplied with antibiotics were carried out with the knockout mutant and two control strains, one harboring plasmid pCOLADuet-1::MVA1–5::$cphA_{6308}$ and the other harboring a conventional expression plasmid pET-23a::$cphA_{6308}$. As revealed by measuring the colony-forming units of aliquots spread on solid media with or without antibiotics, the knockout mutant revealed a plasmid stability of 100% whereas the control strains exhibited plasmid stabilities of only 64% and 2%, respectively. Radiometric enzyme activity measurements for CphA revealed only 95% and 12.5% of the activity in the control strains harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$ and pET-23a::$cphA_{6308}$, respectively, in comparison to the activity measured in the knockout mutant. The knockout mutant synthesized 9.5% (w/w of cell dry weight (CDW)) of cyanophycin, and the control strain harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$ synthesized 13.6% (w/w of CDW) after growth without antibiotics.

1. Introduction

Biotechnological production processes often rely on plasmids in well-known microorganisms like *Escherichia coli*. Therefore, it is desirable to use plasmids that contain the necessary genes for the desired product and are stably maintained in the cells over many generations. Microorganisms are often exposed to extreme environmental conditions in natural habitats, which require individual and special properties for survival and which were acquired during evolution. In addition, the adaption of metabolic processes to the environment is an important regulation (Gerdes et al., 2005). Addiction systems are also regulatory elements in bacteria, which are apparently used not only to ensure stable plasmid maintenance (Zielenkiewicz and Cegłowski, 2001) but also to regulate the rate of replication and translation in the cell (Gerdes et al., 2005). Decades of research have revealed many such addiction systems and numerous subgroups (Zielenkiewicz and Cegłowski, 2001). To date, many naturally occurring and engineered addiction systems have been described; however, not every system has reached an applicable status. The currently known addiction systems can be divided into three major groups: (i) toxin/antitoxin-based systems (TA systems), (ii) operator/repressor system (Cranenburgh et al., 2001), and (iii) catabolism-based metabolic systems.

* Corresponding author. Corrensstraße 3, D-48149 Münster, Germany. Fax: +49 251 833 8388.
E-mail address: steinbu@uni-muenster.de (A. Steinbüchel).

1096-7176/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2009.01.007

Addiction systems can be used biotechnologically for different purposes. The contamination of the environment with bacteria used for biotechnological processes can be prevented by cloning a gene encoding a toxin collinear to the promoter of another operon, which is activated by the limitation of a substrate that is not naturally present outside the laboratory (Schweder et al., 1992). One example of using addiction systems for biotechnological purposes is to increase the yield of the product by stabilizing the plasmid in the cells, which encodes the enzymes for this catabolic pathway with an addiction system (Voß and Steinbüchel, 2006). This aspect and the advantage of needing no additional antibiotics are important factors for industry to use such established addiction systems as a high performance production platform. Nevertheless, none of the mentioned systems resulted in complete plasmid stability due to several problems, which will be discussed later.

Two isoprenoid biosynthesis pathways are known that synthesize the unique isoprenoid precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). The mevalonate (MVA)-dependent pathway is used by most eukaryotes to convert acetyl-coenzyme A to IPP and DMAPP, whereas most prokaryotes use the deoxyxylulose 5-phosphate (DXP) pathway for isoprenoid biosynthesis starting from pyruvate and glyceraldehyde 3-phosphate (Fig. 1) (Boucher and Doolittle, 2000). Plants and most algae, except for the green algae, possess both the MVA pathway in the cytoplasm and the DXP pathway in the plastids (Kuzuyama, 2002). The demand of cells for isoprenoids and especially of the precursor IPP is essential for survival. One example is the need of IPP for tRNAs prenylation processes (Connolly and Winkler, 1989), and another example is the synthesis of farnesyl pyrophosphate (FPP), which is involved in cell wall biosynthesis, from IPP. Based on these facts, a new class of anabolic addiction systems was established. We chose the *ispH* gene of the DXP pathway as a central element of our addiction system, because IspH mediates solely the last step of IPP biosynthesis in *E. coli* and is essential for survival (McAteer et al., 2001). As an application, we visualized the outstanding plasmid stability of our addiction system with coupling to cyanophycin production in *E. coli*.

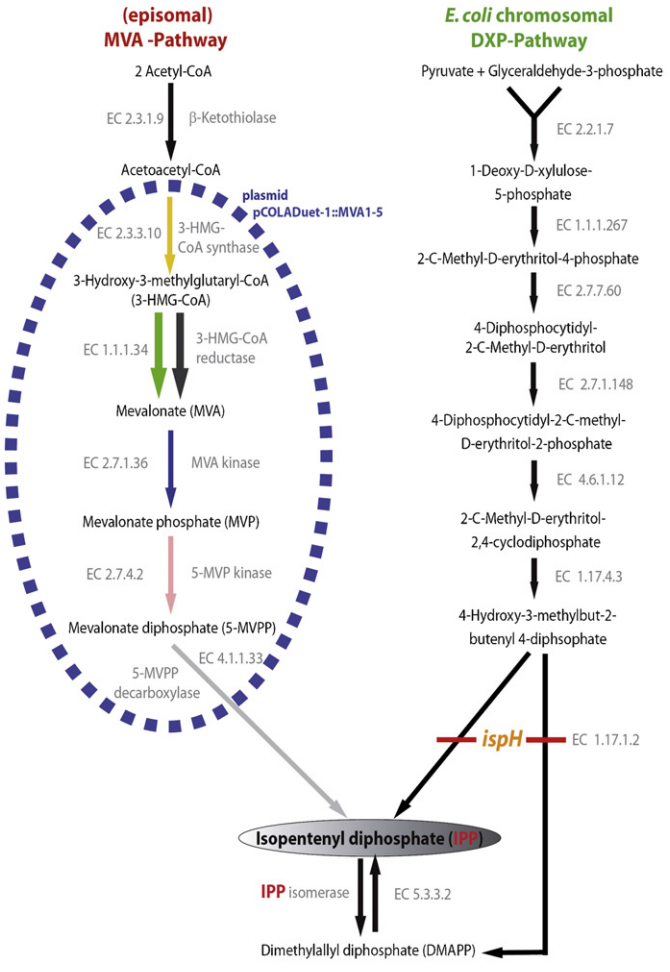

Fig. 1. Strategy and principle of the novel anabolism-based Δ*ispH*/MVA addiction system. The two independent DXP and MVA pathways, which result both in the final product isopentenyl diphosphate starting from different substrates, are shown. The wild type of *E. coli* harbors only the DXP pathway. This pathway is inactive in the knockout mutant *E. coli* HMS174(DE3) Δ*ispH* Ω FRT/Amp/FRT. IPP biosynthesis is conferred again to this mutant by the synthetic episomal MVA pathway encoded by plasmid pCOLADuet-1::MVA1–5 or derivatives, thereby solely allowing the recombinant *E. coli* strains to synthesize IPP.

Cyanophycin, also referred to as multi-L-arginyl-poly(L-aspartic acid) or CGP, represents a ribosome-independently synthesized polypeptide consisting of a poly(aspartic acid) backbone with arginine moieties linked to the β-carboxyl group of each aspartate by its α-amino group (Simon and Weathers, 1976). The ability for CGP synthesis is widely distributed among cyanobacteria, and also some heterotrophic bacteria were shown to synthesize the polymer naturally (Krehenbrink et al., 2002; Elbahloul et al., 2005). Several bacteria and few eukaryotic organisms were applied for recombinant expression of the key enzyme cyanophycin synthetase in heterotrophic bacteria and in eukaryotic organisms (Aboulmagd et al., 2001; Elbahloul et al., 2005; Neumann et al., 2005; Voss and Steinbüchel, 2006; Hai et al., 2006; Steinle et al., 2008). In recombinant *E. coli* 34.5% (w/w) of CDW were accumulated at the maximum using a truncated *cphA* (Hai et al., 2006), whereas CGP contents of up to 46.0% (w/w) were obtained in *A. baylyi* ADP1 after medium optimization (Elbahloul and Steinbüchel, 2006). However, problems concerning the maintenance of stable CGP synthesis still need to be overcome; this will be essential for its biotechnical production (Voss and Steinbüchel, 2006). The poly(amino acid) cyanophycin is of biotechnical and medical interest due to its putative application as precursor for bulk chemicals (Sanders et al., 2007; Scott et al., 2007), as source for biodegradable poly(aspartic acid) (Schwamborn, 1998) and Asp-Arg dipeptides (Sallam and Steinbüchel, 2008).

In this study, we established a novel anabolism-related addiction system based on the mevalonate-independent DXP pathway for IPP biosynthesis. This outstanding system confers practically full plasmid stability to the host organism *E. coli* producing cyanophycin investigated in this study.

2. Material and methods

2.1. Bacterial strains, plasmids, oligonucleotides, and culture conditions

Bacteria and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C in Lysogeny broth (LB) medium (Sambrook et al., 1989). To select plasmid-carrying strains, antibiotics were added to the media at the following concentrations (μg/ml): ampicillin (100), kanamycin (50), rifampicin (34), and tetracycline (12.5).

2.2. DNA manipulation

Digestions of DNA with restriction endonucleases (New England Biolabs, Ipswitch, USA) were done under the conditions

Table 1
Bacteria and plasmids used in this study.

Bacterial strains and plasmids	Relevant characteristics	Reference or source
Bacterial strains		
Escherichia coli HMS174(DE3)	F⁻ *rec*A1 *hsd*R(rK12⁻ mK12⁺) (Rif^R) λDE3	Lucigen (Middelton, USA)
Escherichia coli HMS174(DE3) pCOLADuet-1::MVA1–5	F⁻ *rec*A1 *hsd*R(rK12⁻ mK12⁺) (Rif^R) λDE3 (Km^R)	This study
Escherichia coli HMS174(DE3) Δ*isp*H ΩAmp^R pCOLADuet-1::MVA1–5	F⁻ *rec*A1 *hsd*R(rK12⁻ mK12⁺) (Rif^R) λDE3 (Km^R) (Amp^R)	This study
Escherichia coli HMS174(DE3) pCOLADuet-1::MVA1–5::*cphA*$_{6308}$	F⁻ *rec*A1 *hsd*R(rK12⁻ mK12⁺) (Rif^R) λDE3 (Km^R) *cphA*$_{6308}$ coding for CphA$_{6308}$ with point mutation C595S	This study
Escherichia coli HMS174(DE3) Δ*isp*H ΩAmp^R pCOLADuet-1::MVA1–5::*cphA*$_{6308}$	F⁻ *rec*A1 *hsd*R(rK12⁻ mK12⁺) (Rif^R) λDE3 (Km^R) (Amp^R) *cphA*$_{6308}$ coding for CphA$_{6308}$ with point mutation C595S	This study
Plasmids		
pET-23a	Amp^R, T7-promoter	Novagen (Darmstadt Germany)
pBBR1MCS-2::*cphA*$_{6308}$	Kan^R, *lac*-promoter	Aboulmagd et al. (2001)
pET-23a::*cphA*$_{6308}$	Amp^R, T7-promoter *cphA*$_{6308}$ coding for CphA$_{6308}$ with point mutation C595S	This study
pCOLADuet-1	Km^R, T7/*lac*-promoter	Novagen (Darmstadt Germany)
pCOLADuet-1::MVA1–5	Km^R, T7/*lac*-promoter	This study
pCOLADuet-1::MVA1–5::*cphA*$_{6308}$	Km^R, T7/*lac*-promoter *cphA*$_{6308}$ coding for CphA$_{6308}$ with point mutation C595S	This study

described by Sambrook et al. (1989) or by the manufacturer. *Pfx*-DNA polymerase (Invitrogen, Karlsruhe, Germany) and other DNA-manipulating enzymes were used as described by the manufacturers. Primers were purchased from Thermo Fisher Scientific AG (Waltham, USA). DNA fragments were isolated from agarose gels by using the E.Z.N.A. Gel Extraction kit (Omega Bio-Tek Inc., Doraville, USA).

2.3. Transfer of plasmids

Plasmids were transferred into *E. coli* by employing the $CaCl_2$ method (Sambrook et al., 1989). If necessary, plasmids were transferred by electroporation before applying the integration and recombination protocol of the "*E. coli* Gene Deletion Kit" with all parameters as described by the manufacturer (Genebridges GmbH, Dresden, Germany).

2.4. DNA sequence analysis

DNA sequences were determined with a model 4000L DNA sequencer (LI-COR Inc., Biotechnology Division, Lincoln, NE, USA) and a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Epicentre Technologies, Madison, USA) according to the instructions of the manufacturer.

2.5. Generation of a site-directed mutagenized cphA

For generation of a site-directed mutagenized *cphA* gene from *Synechocystis* sp. PCC 6308, the fusion PCR technique was applied using the oligonucleotides #7 and #8 as primers and the vector pBBR1MCS-2::*cphA*$_{6308}$ as template (Horton et al., 1989). The mutagenized *cphA* gene was cloned into the expression vector pET-23a; recombinant *E. coli* BL21(DE3) cells harboring CphA$_{6308}$ with point mutation C595S exhibited increased CphA enzyme activity and also a 20% higher CGP content. In this study only the mutated CphA with point mutation C595S was used; the corresponding gene was designated *cphA*$_{6308}$ throughout in the manuscript.

2.6. Construction and cloning of the synthetic MVA1–5 cluster

To construct plasmid pCOLADuet-1::MVA1–5, a two-step procedure was chosen: (i) the two hybrid plasmids pCOLADuet-1::MVA-top and pCOLADuet-1::MVA-bottom were synthesized and sequence optimized for the codon usage of *E. coli* (BioBasic Inc., Ontario, Canada). (ii) Both plasmids were then enzymatically digested with *Kpn*I plus *Mfe*I, yielding a 2103-bp DNA fragment, which was ligated into pCOLADuet-1::MVA-top, yielding pCOLADuet-1::MVA1–5. The nucleotide sequence of the entire plasmid was verified by sequencing (GATC Biotech, Konstanz, Switzerland). The respective genes were chosen from these organisms as their gene products were shown to be functionally active (http://www.brenda-enzymes.info/).

2.7. Cloning of cphA$_{6308}$ into the MVA1–5 cluster (Fig. 2A)

The previously received hybrid plasmid pCOLADuet-1::MVA1–5 was digested and linearized with *Xho*I. The constructed pET-23a::*cphA*$_{6308}$ hybrid plasmid served as PCR template to amplify the T7 promoter region and *cphA*$_{6308}$ with 5′ and 3′ *Xho*I restriction sites. For this, we used primers #1 and #2 in a standard PCR protocol. The obtained PCR fragment was isolated and ligated into the linearized pCOLADuet-1::MVA1–5 and yielded pCOLADuet-1::MVA1–5::*cphA*$_{6308}$. The correct sequence of *cphA*$_{6308}$ and orientation was verified by DNA sequence analysis.

2.8. Construction of the chromosomal ispH knockout in E. coli HMS174(DE3)

For generating a genomic knockout of *ispH* in *E. coli* HMS174(DE3), the "*E. coli* Gene Deletion Kit" (Genebridges GmbH, Dresden, Germany) was used. Employing primers #3 and #4 (Table 2) and the FRT-gb2-amp-FRT template (Genebridges GmbH, Dresden, Germany), which contains an FRT recognition sequence flanking the gb2 promoter followed by an ampicillin resistance gene, a PCR product with *ispH* homologous flanking regions of 50 bp at the 5′ and 3′ regions was obtained. This was done in a two-step PCR using AccuPrime *Pfx* polymerase (Invitrogen, Carlsbad, USA) with the denaturating step (15 s) at 95 °C, the elongation step (2 min) at 68 °C and 33 repeats. A 1.8 kbp fragment was obtained, separated by agarose gel electrophoresis and then extracted from the gel (Qiagen GmbH, Hilden, Germany). Next steps were carried out according to the instructions provided by the manufacturer of the kit, and the obtained clones were incubated at 37 °C on LB agar plates with appropriate antibiotics (ampicillin: 100 μg/ml, rifampycin: 34 μg/ml, kanamycin: 50 μg/ml).

2.9. Analysis of plasmid stability

To investigate plasmid stability, samples were withdrawn from the cultivation vessels at the assigned time, and aliquots were spread onto LB agar plates containing or not containing kanamycin. The plates were incubated for 24 h at 37 °C, and the

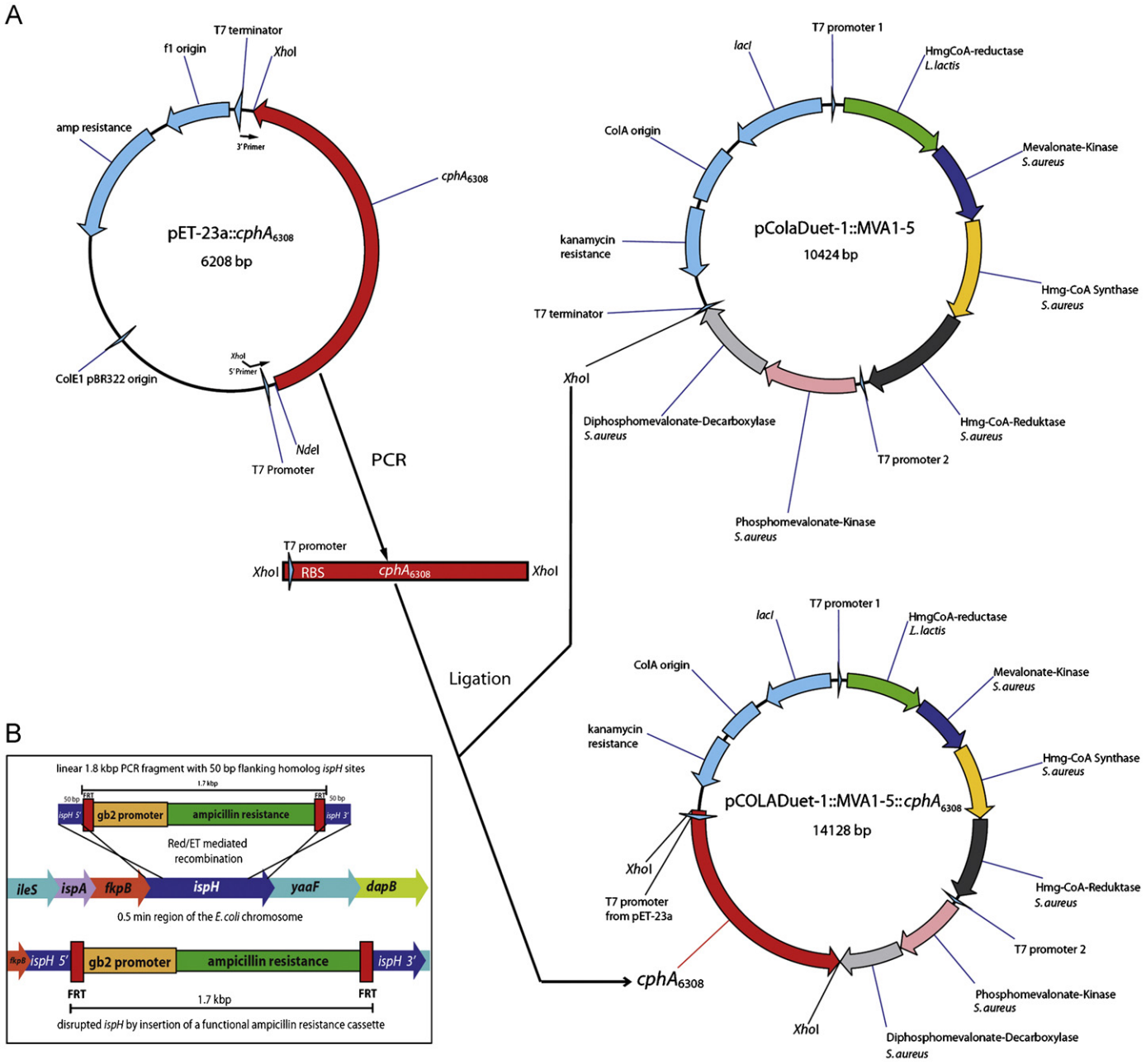

Fig. 2. Scheme of the cloning strategy and chromosomal knockout. (A) Cloning scheme and construction of plasmids. Plasmid pET-23a::$cphA_{6308}$ was used as the PCR template to amplify a 2.8-kbp fragment with *Xho*I/*Xho*I restriction sites comprising $cphA_{6308}$. After digestion of this PCR product with *Xho*I, it was ligated into the *Xho*I restriction site of plasmid pCOLADuet-1::MVA1–5, which functions as a platform for an episomal MVA pathway. Finally, the resulting plasmid pCOLADuet-1::MVA1–5::$cphA_{6308}$ was used to demonstrate the functionality of the addiction system by product analysis and plasmid stability tests. (B) Chromosomal knockout of *ispH* in *E. coli* and integration of a functional selection cassette. A linear 1.8-kbp PCR fragment containing a functional ampicillin resistance gene cassette with 50 bp 5′ and 3′ flanking *ispH* homologous sequences for selection was amplified. This fragment was integrated into *ispH* by a Ret/ET mediated recombination procedure. The *E. coli* knockouts were selected on ampicillin-containing media.

colony-forming units (CFU) were determined and analyzed by comparing the CFU on LB agar plates without kanamycin with the CFU on LB agar plates containing kanamycin.

2.10. Transmission electron microscopic studies

Cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 45 min. After three washes with PBS (20 min each), cells were postfixed in 1% (w/v) osmium tetroxide in 0.1 M PBS (pH 7.3) and washed once with PBS for 20 min. Then the water was removed by using a graded water/ethanol series (30%, 50%, 70%, 90%, 96% and absolute ethanol) with each step lasting about 15 min. To obtain thin sections, the samples were embedded in Spurr resin without propylene oxide (Spurr, 1969). Sections with a thickness of 70–80 nm were cut with an ultramicrotome (Leica Mikroskopie und Systeme, Wetzlar, Germany) by using a diamond knife and were placed on a 200-mesh copper grid. Subsequently, the sections were stained with saturated uranyl acetate solution for 30 min and with lead citrate

Table 2
Oligonucleotides used in this study.

No. #	Oligonucleotide	Nucleotide sequence (5′–3′)
1	pET23aXhoIT7_fw	5′-AACTCGAGATTAATACGACTCACTATAGG-3′
2	T7Terminator	5′-GCTAGTTATTGCTCAGCGG-3′
3	ispH_integr_fw	5′-ATGCAGATCCTGTTGGCCAACCCGCGTGGTTTTTGTGCCGGGGTAGACCGAATTAACCCTCACTAAAGGGCGGC-3′
4	ispH_integr_rev	5′-TTCACGAATATCGACACGCAGCTCTTTCGGCACTTCGAAAACAATGTTTTTAATACGACTCACTATAGGGCTC-3′
5	5′-*cphA*6308-*Nde*I	5′-GCGGCATATGAAAATCCTCAAAACACAAAC-3′
6	3′-*cphA*6308-*Xho*I	5′-GCGCCTCGAGCTATTCACTACTGAGATG-3′
7	C595S_fw	5′-CCTTTGAAAGCTCTGATGTTGGCG-3′
8	C595S_rev	5′-CGCCAACATCAGAGCTTTCAAAGG-3′
9	5′_ins_contr	5′-CCAACCCGCGTGGTTTTTGT-3′
10	3′_ins_contr	5′-ACACGCAGCTCTTTCGGCAC-3′

solution according to Reynolds (1963) for 3 min. Imaging was performed with an H-500 transmission electron microscope (Hitachi, Tokyo, Japan) in the bright-field mode at an acceleration voltage of 80 kV at room temperature.

2.11. Cell harvest, determination of cell dry matters, and cell disruption

Cells of *E. coli* were harvested with a bench centrifuge (15 min, 2800*g*, 4 °C), and cell pellets were washed once with 0.9% NaCl (w/v). To determine the cell dry weight (CDW), pellets were lyophilized, and the cell dry mass was gravimetrically determined. For cell disruption, the cell pellet was resuspended in 1 ml buffer (20 mM Tris–HCl, pH 7.5) per g fresh or dry cell mass and disrupted for 5 min by a bead mill (Type MM 301, Retsch, Haan, Germany). Soluble cell fractions were obtained by centrifugation of crude cell extracts (10 min, 16,000*g*, 4 °C).

2.12. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 11.5% (w/v) gels as described by Laemmli (1970). Staining of proteins and CGP was performed with Serva Blue R. Protein concentrations were determined as described by Hartree (1972). Molecular weight standard proteins were purchased from Fermentas; the applied mixture contained β-galactosidase from *E. coli* (117 kDa), bovine serum albumin from bovine plasma (90 kDa), ovalbumin from chicken egg white (49 kDa), carbonic anhydrase from bovine erythrocytes (35 kDa), β-lactoglobulin from bovine milk (26 kDa), and lysozyme from chicken egg white (19 kDa).

2.13. Isolation and determination of cyanophycin

Cyanophycin was isolated in a small-scale from lyophilized cells grown in 50 ml LB medium after the acid-extraction method described by Frey et al. (2002). The composition of the polymer was determined by HPLC as described previously (Aboulmagd et al., 2000) using an OPA column B801 (Waters, Milford, MA, USA). Calibrations were carried out with samples from an amino acid reference kit (Kollektion AS-10 from Serva Feinbiochemica, Heidelberg, Germany).

2.14. Cyanophycin synthetase assay

Cyanophycin synthetase activity was determined by a radiometric assay according to the protocol described previously (Aboulmagd et al., 2000). Soluble cell fractions were used to determine the enzyme activity. Scintillation counting was carried out in a model LS 6500 scintillation counter (Beckman Instruments GmbH, München, Germany).

3. Results

3.1. Principle of MVA/ΔispH-based addiction system in E. coli

To construct the MVA/Δ*ispH* addiction system we used *E. coli* HMS174(DE3) as an appropriate host strain because of the chromosomal rifampycin resistance and T7 RNA polymerase gene (DE3) in the following accomplished procedures. The principle of this anabolism-based system is shown in Fig. 1. The DXP pathway, which starts from pyruvate and glyceraldehyde-3-phosphate, and the (artificially introduced) MVA pathway, which starts from acetyl-CoA, result both in the final product (IPP) that is required for biosynthesis of a large variety of essential cell constituents in any organism. The wild type of *E. coli* harbors only the DXP pathway. This pathway is inactive in the knockout mutant *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5 Δ*ispH* ΩFRT/Amp/FRT. IPP biosynthesis is conferred to this mutant by the synthetic episomal MVA pathway encoded by plasmid pCOLADuet-1::MVA1–5 or derivatives, thereby solely allowing the recombinant *E. coli* strains harboring and maintaining this plasmid to synthesize IPP. Therefore, the preferred gene(s) for the desired biotechnological application should be cloned into this plasmid. We could demonstrate that no isopropyl-β-D-thiogalactopyranosid (IPTG) induction is necessary depending on the sufficient basal expression level by the used T7 promoter. The generation of the addiction system with a gene of choice is described in Sections 3.2–3.4. It is important that the steps are done in the described order.

3.2. Establishment of the episomal MVA pathway in E. coli

In the first step we designed a hybrid plasmid, which was referred to as pCOLADuet-1::MVA1–5 and which harbors all necessary genes from *Lactococcus lactis* or *Staphylococcus aureus* to constitute a synthetic mevalonate pathway in *E. coli*. These organisms represent exceptions among prokaryotes because they possess the MVA pathway. The respective genes were chosen from these organisms as their gene products largely showed to be functionally active (http://www.brenda-enzymes.info/) in *E. coli*. To circumvent the well-known bottleneck of HMG-CoA reductase activity as the rate-limiting step in the MVA pathway (Bochar et al., 1999), we cloned a second and different HMG-CoA reductase in our platform plasmid. Moreover, the applied genes were codon-optimized for *E. coli* to enable an optimal heterologous expression.

3.3. Integration of cyanophycin synthetase gene into pCOLADuet-1::MVA1–5

Secondly, to link cyanophycin biosynthesis with this synthetic MVA pathway and to establish a stable platform for cyanophycin

synthesis, we decided to clone *cphA* of *Synechocystis* sp. PCC 6308, harboring the point mutation C595S from pET-23a::$cphA_{6308}$, into pCOLADuet-1::MVA1–5, resulting in plasmid pCOLADuet-1::MVA1–5::$cphA_{6308}$. After successful transformation of *E. coli* HMS174(DE3) with pCOLADuet-1::MVA1–5::$cphA_{6308}$, the obtained recombinant strain *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5::$cphA_{6308}$ was cultivated with kanamycin to force plasmid stability. Moreover, plasmid pET-23a::$cphA_{6308}$ was also transferred to *E. coli* HMS174(DE3) and applied as control strain not expressing genes coding for the MVA pathway episomally.

3.4. Generation of a chromosomal ΔispH mutant of E. coli

In the third step and to generate a Red/ET-mediated *ispH*/*lytB* knockout mutant, we used *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5::$cphA_{6308}$ and *E. coli* HMS174(DE3) pCOLADuet-1 as the control strain. After the recombination process, about 320 colonies occurred on LB agar plates containing ampicillin as selection marker for the possible integrated functional FRT/Amp/FRT cassette in *ispH* of the recombinant strain *E. coli* HMS174(DE3) carrying the mevalonate cluster and cyanophycin synthetase. The chromosomal knockout of *ispH* was verified by PCR with primers #9 and #10 and subsequently by DNA sequencing of the PCR product. The used control strain without pCOLADuet-1::MVA1–5::$cphA_{6308}$ did not yield transformants. The recombination principle and chromosomal knockout location is visualized in Fig. 2B. *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5::$cphA_{6308}$ with intact chromosomal *ispH* gene was further applied as the control strain.

3.5. Growth and plasmid stability of the cells

To demonstrate the functionality of the established addiction system, it was applied to biosynthesis and production of cyanophycin (see Section 3.3). Cyanophycin production is easily monitored by examination of cell and colony morphology. Furthermore, convenient methods for isolation of the polymer from the cells and for its reliable quantitative analysis were developed previously (Frey et al., 2002). In addition to the knockout mutant harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$, *E. coli* HMS174(DE3) harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$ and *E. coli* HMS174(DE3) harboring pET-23a::$cphA_{6308}$ were examined as control strains not possessing this addiction system. Cyanophycin biosynthesis and plasmid stability were examined in all strains applying a variety of methods (Section 3.6).

Experiments monitoring cell growth and plasmid stability were carried out in flask experiments. The cells were grown in a medium supplied or not supplied with appropriate antibiotics. The medium was inoculated with 1% (v/v) of a preculture in 100 ml LB medium without antibiotics. Growth of the cells was monitored by determining the optical density in a Klett–Summerson colorimeter. As shown in Fig. 3, both control strains revealed a slightly faster and stronger growth than the knockout mutant.

For verification of plasmid stabilities, cultures were grown for 24 h at 37 °C, appropriately diluted and spread onto LB agar plates to count colony growth in the presence or absence of antibiotics. The obtained colonies are shown in Fig. 4. A plasmid loss of 98% was determined for the control strain harboring pET-23a::$cphA_{6308}$, and the loss was 36% for the control strain harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$. In contrast, the knockout mutant exhibited a plasmid stability of 100%. Furthermore, in the strain with intact *ispH* gene, only 64% of plasmid pCOLADuet-1::MVA1–5::$cphA_{6308}$ was detected in comparison to the *ispH* knockout mutant. Colony morphology of the strains harboring

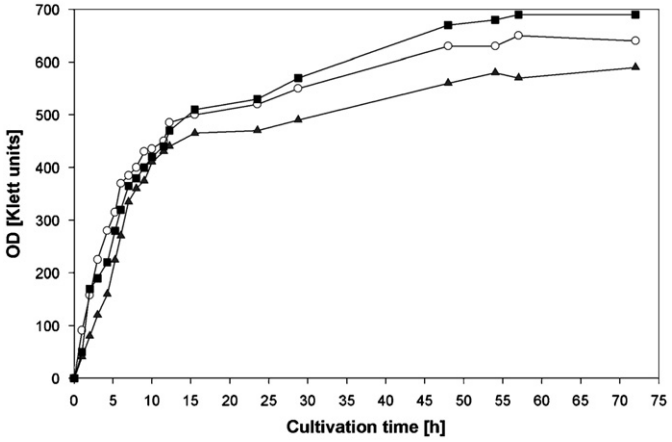

Fig. 3. Growth of recombinant *E. coli* cells with and without Δ*ispH*/MVA addiction system. *E. coli* HMS174(DE3) pET-23a::$cphA_{6308}$ (filled squares), *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5 (circles) and *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5 Δ*ispH* ΩFRT/Amp/FRT (filled triangles) were cultivated for 72 h in 250 ml baffled Klett flasks each containing 100 ml LB medium without antibiotics at 30 °C. The cultures were inoculated with 1% (v/v) of a preculture grown in the presence of appropriate antibiotics (rifampicin: 30 μg/ml, kanamycin: 50 μg/ml, ampicillin: 100 μg/ml) in the exponential growth phase cultivated for 12 h under the same conditions as described before. The optical density was measured with a Klett–Summerson colorimeter (Filter no. 54; Manostat Corporation, Cat. no. 76-550-220, USA) at 520 nm.

pCOLADuet-1::MVA1–5::$cphA_{6308}$ also indicated plasmid loss: the knockout mutant formed white, opaque colonies, thereby indicating cyanophycin accumulation in the cells, whereas both control strains formed also a more or less large fraction of translucent colonies (Fig. 5).

3.6. Cyanophycin synthetase activity, cyanophycin content of the cells, and analysis of cell extracts by SDS-PAGE

To verify the accumulation of cyanophycin in strains grown in the presence or absence of antibiotics, the investigated strains were cultivated as described above, and CphA enzyme activity and cyanophycin contents were determined; in addition, microscopic pictures and analysis of cell extracts by SDS-PAGE were performed. All applied strains revealed significant specific enzyme activities of up to 0.81 U/mg protein when grown in medium containing appropriate antibiotics (Table 3). After growth in the medium not supplied with antibiotics, *E. coli* HMS174(DE3) harboring pET-23a::$cphA_{6308}$ exhibited a strongly diminished specific CphA enzyme activity of only 0.04 U/mg protein. This observation was consistent with the observation that no detectable cyanophycin could be isolated from these cells (Table 3). The control strain harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$ synthesized the highest amount of cyanophycin and revealed the highest CphA enzyme activity, whereas a decrease in polymer content and in enzyme activity was observed after growth in the absence of antibiotics. In contrast, the knockout mutant stably synthesized 9.40% or 9.52% (w/w of CDM) cyanophycin and revealed an enzyme activity of 0.41 or 0.44 U/mg protein, independent of whether the cells were cultivated in the presence or absence of antibiotics, respectively. HPLC analysis revealed that the isolated polymers consisted of 50 mol% aspartic acid, 44–46 mol% arginine, and 4–6 mol% lysine.

Furthermore, the results presented above were consistent with light microscopic examinations of the cells after growth in the absence of antibiotics (Fig. 6). Whereas all cells of the knockout mutant possessed light-scattering inclusions indicating the presence of cyanophycin granules (Fig. 6A), the inclusions were frequently absent in cells of the control strain not harboring the

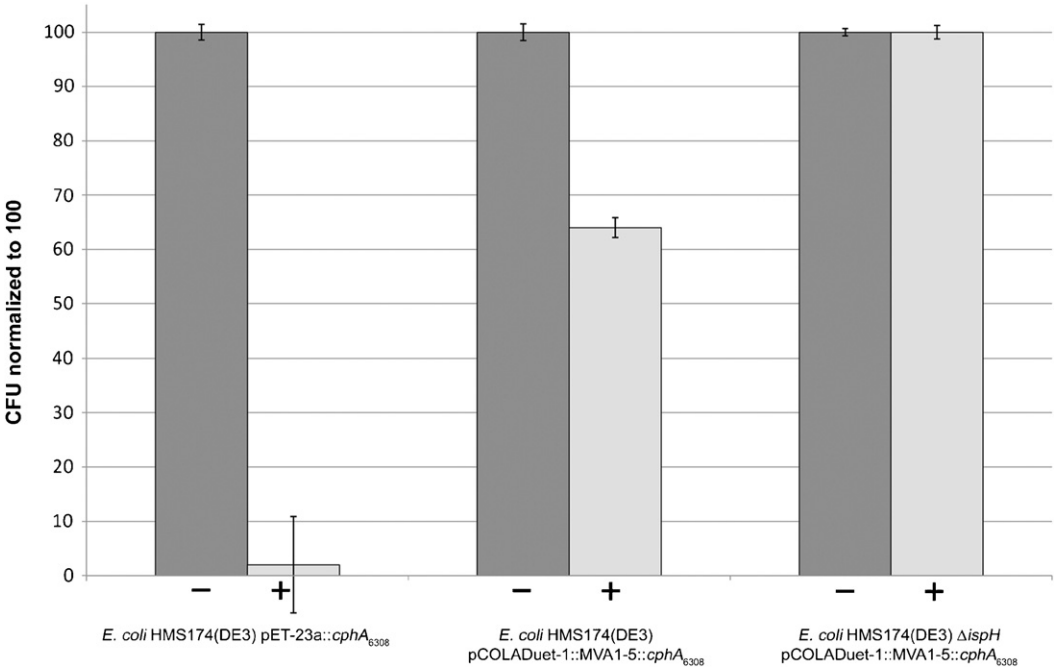

Fig. 4. Determined plasmid loss of recombinant *E. coli* cells with and without a Δ*ispH*/MVA addiction system. The used strains *E. coli* HMS174(DE3) pET-23a::$cphA_{6308}$, *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5::$cphA_{6308}$ and *E. coli* HMS174(DE3) Δ*ispH* ΩFRT/Amp/FRT pColaDuet-1::MVA1–5::$cphA_{6308}$ were inoculated with 1% (v/v) of a 12 h growing preculture in LB medium with appropriate antibiotics and inoculated in a baffled 250 ml flask with 100 ml LB media without antibiotics at 180 rpm at 30 °C. To determine the plasmid stability, samples were withdrawn from the cultivation vessels after 72 h (compare to Fig. 3). Aliquots of 100 μl of these samples were appropriately diluted and spread onto LB agar plates containing or not containing kanamycin or ampicillin. The plates were incubated for 16 h at 37 °C, and the colony-forming units (CFU) were counted. The received values obtained for LB agar plates without antibiotics (−) were set as 100%, and the % CFU values obtained in LB agar plates plus kanamycin (+) were related to the latter. Standard deviations are represented by vertical error bars.

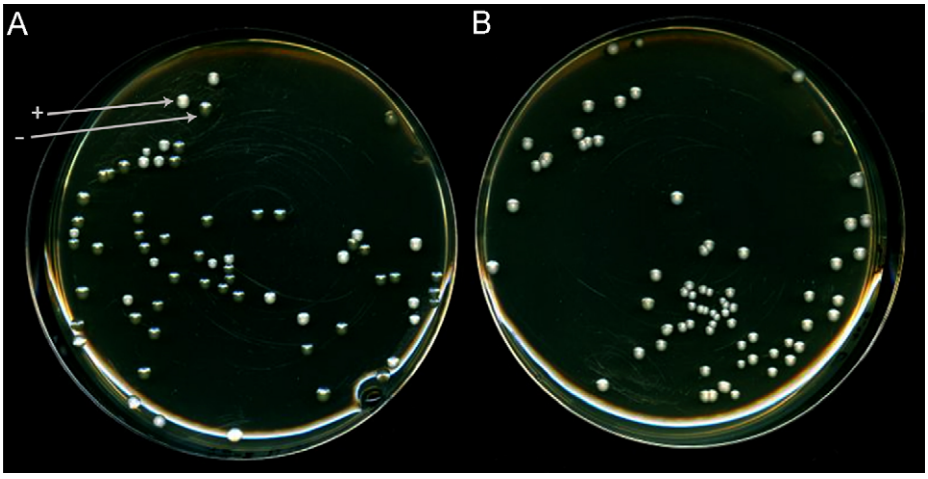

Fig. 5. Visible plasmid loss of recombinant cyanophycin-producing *E. coli* cells without (A) and with (B) Δ*ispH*/MVA addiction system. Appropriate volumes of diluted cell suspensions were applied to LB agar plates after 24 h of cultivation in LB medium at 37 °C without antibiotics. Accumulation of cyanophycin resulted in an enhanced opacity of the colonies (“white cells”, arrow marked with “+”). On the other hand, cells that had lost the plasmid harboring *cphA* yielded normal translucent colonies (arrow marked with “−”).

ispH knockout (Fig. 6B). To further confirm the assumption that the light-scattering inclusions occurring in the cells of the deletion mutant are cyanophycin, electron microscopic pictures were taken. These pictures showed widely distributed electron-dense black areas in the cells of this strain, which show intracellular cyanophycin granules (Fig. 7A and B). In contrast, cells of the *E. coli* strain harboring pET-23a::$cphA_{6308}$ did not harbor any inclusions (Fig. 6C).

All these observations were fully supported by the analyses of cell extracts by SDS-PAGE (Fig. 8). Large diffuse regions, corresponding to the widely polydisperse cyanophycin, were observed with the cell raw extracts of cells that were grown under selective pressure (Fig. 8A), whereas such regions were absent in lanes loaded with cell extracts of strains harboring pET-23a::$cphA_{6308}$ grown in the absence of ampicillin. On the other hand, cells of both strains harboring plasmid pCOLADuet-1::MVA1–5::$cphA_{6308}$ yielded these regions representing cyanophycin after growth in the absence of kanamycin (Fig. 8B).

4. Discussion

Conventional plasmid expression systems based on antibiotic selection markers often face the problem that the respective

Table 3
Specific CphA enzyme activity and CGP content in recombinant cells of *E. coli* HMS174(DE3) harboring *cphA* from *Synechocystis* sp. PCC 6308 with point mutation C595S after growth in LB medium for 24 h with or without addition of the appropriate antibiotic.

E. coli HMS174(DE3) with plasmid	Condition	Spec. enzyme activity (U/mg)	CGP content of cells (%, w/w, of CDW)
Δ*ispH* pCOLADuet-1::MVA1–5::$cphA_{6308}$	With antibiotic	0.41±0.1	9.40±1.2
pCOLADuet-1::MVA1–5::$cphA_{6308}$	With antibiotic	0.81±0.1	16.08±2.0
pET-23a::$cphA_{6308}$	With antibiotic	0.65±0.1	10.20±0.5
Δ*ispH* pCOLADuet-1::MVA1–5::$cphA_{6308}$	Without antibiotic	0.44±0.1	9.52±1.0
pCOLADuet-1::MVA1–5::$cphA_{6308}$	Without antibiotic	0.70±0.1	13.60±1.0
pET-23a::$cphA_{6308}$	Without antibiotic	0.04±0.1	0.00±0.0

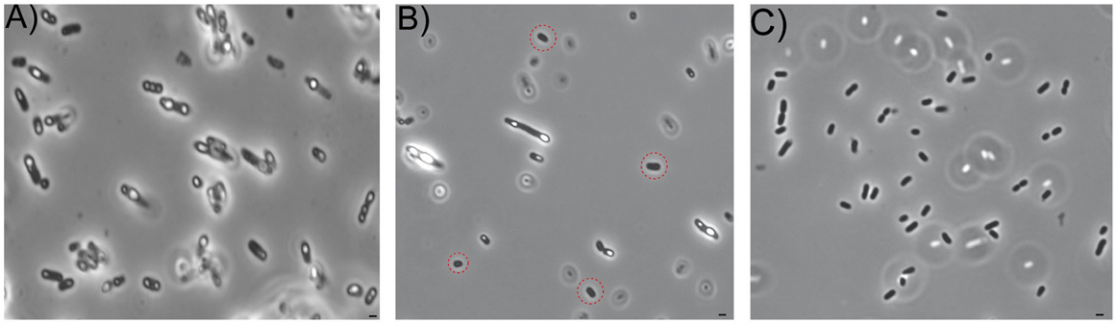

Fig. 6. Light microscopic images of recombinant *E. coli* HMS 174(DE3) after 24 h of cultivation in LB medium without antibiotics. The images of the recombinant *E. coli* strains were taken with an AXIO IMAGER M1 phase contrast light microscope and Axio Vision software (Carl Zeiss AG, Göttingen, Germany). The bars represent a length of 1 μm. (A) Cells of *E. coli* HMS 174(DE3) pCOLADuet-1::MVA1–5::$cphA_{6308}$ *ΔispH* ΩFRT/Amp/FRT possessing the *ΔispH*/MVA addiction system. (B) Cells of *E. coli* HMS174(DE3) harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$. (C) Cells of *E. coli* HMS174(DE3) pET-23a::$cphA_{6308}$.

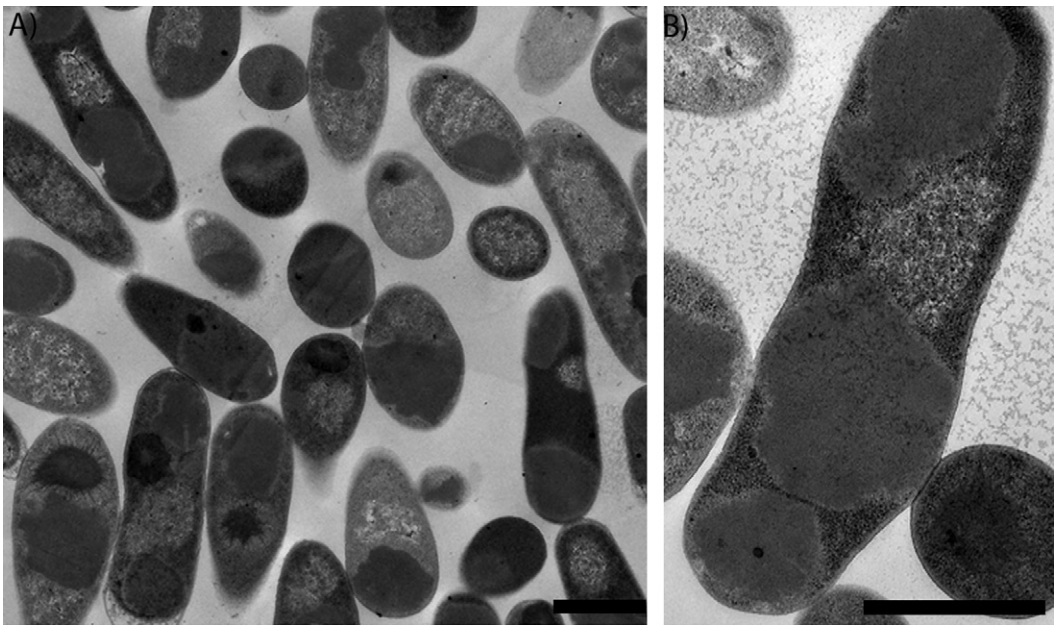

Fig. 7. Transmission electron microscopic (TEM) images of thin sections of *E. coli* HMS174(DE3) pCOLADuet-1::MVA1–5::$cphA_{6308}$ Δ*ispH* ΩFRT/Amp/FRT after 24 h (A+B). The bar represents a length of 0.5 μm.

antibiotic is degraded or modified by the mediating resistance factor especially during long-time fed-batch or high cell density cultivation processes. Consequently, cells not harboring the plasmid have an advantage in growth and in accomplishing the total cell number against the plasmid-carrying cells. One possible solution of this problem is the application of so-called addiction systems that function without a need for an antibiotic in several ways. During over 40 years of research, many natural and synthetic addiction systems have been discovered and constructed (Zielenkiewicz and Cegłowski, 2001). Not every addiction system has been advanced to an applicable status. Naturally occurring addiction systems can be applied biotechnologically in different ways. The largest group of known addiction systems are toxin/antitoxin-based systems. Three different toxin/antitoxin systems are described in the literature: (i) antisense-RNA-regulated loci, (ii) protein-regulated loci, and (iii) restriction

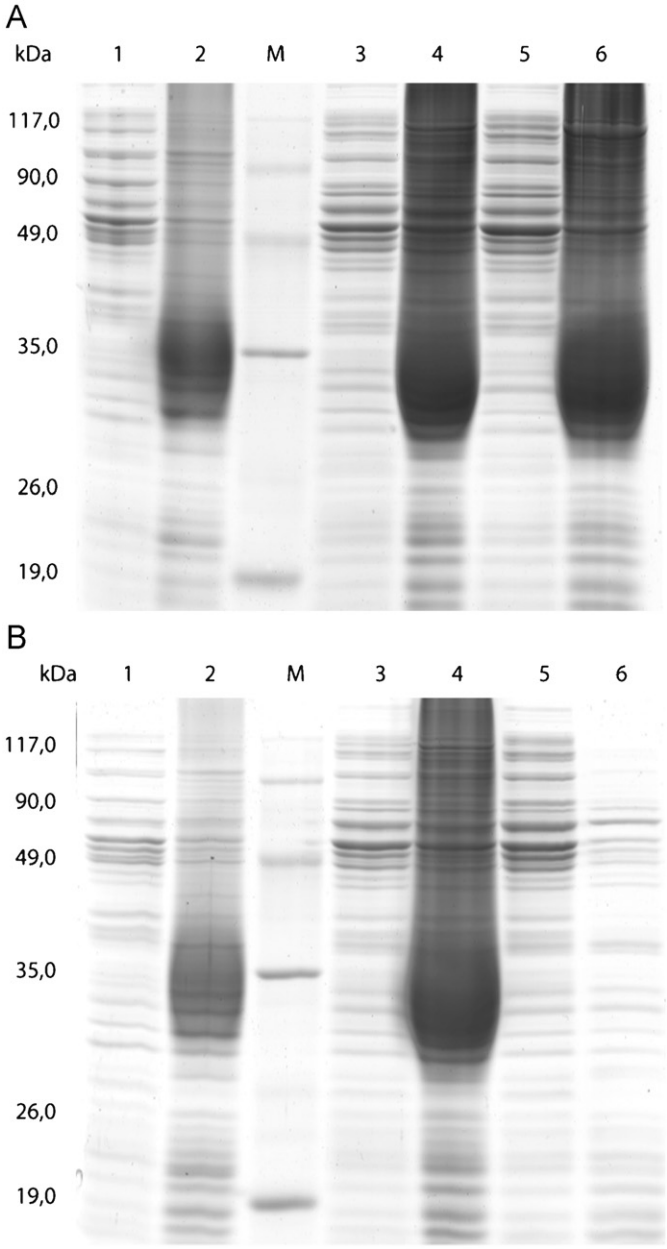

Fig. 8. Analysis of soluble cell fractions and cell crude extracts by SDS polyacrylamide gel electrophoresis. Analysis of soluble cell fractions and cell crude extracts of different recombinant *E. coli* HMS174(DE3) strains grown in the presence or absence of antibiotics. All strains were grown in 50 ml LB medium for 24 h at 37 °C. Cell crude extracts were obtained after cell disruption, soluble cell fractions were obtained by centrifugation of cell crude extracts. (A) Extracts of cells grown in the presence of the appropriate antibiotic. (B) Extracts of cells grown without the addition of antibiotics. Lanes 1, 3, and 5: soluble cell fractions; lanes 2, 4, and 6: cell crude extracts. Lanes 1 and 2, *E. coli* HMS174(DE3) Δ*ispH* harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$: lanes 3 and 4: *E. coli* HMS174(DE3) harboring pCOLADuet-1::MVA1–5::$cphA_{6308}$; *E. coli* HMS174(DE3) harboring pET-23a::$cphA_{6308}$. Protein concentrations were determined according to Hartree (1972). About 70 μg protein was applied from soluble cell fractions, whereas about 50 μg protein was applied from cell crude extracts.

modification systems (Jensen and Gerdes, 1995). Toxin/antitoxin systems function by the counter-acting principle as e.g. of a toxin and an antitoxin (Ogura and Hiraga, 1983). In such systems, the organism is "addicted" to a provided plasmid encoding a stable toxin and an unstable antitoxin. Another form represents a chromosomally encoded toxin, which is inactivated by an antitoxin encoded episomally (Jensen and Gerdes, 1995). Another group comprises metabolism-based addiction systems. However, until now only catabolism-based systems have been described. They depend on the gene for an enzyme essential for a catabolic pathway; the latter is inactivated in the genome, for example by disruption mutagenesis, and a copy of the intact gene is integrated into the plasmid that also encodes the genes for formation of the desired product (Voß and Steinbüchel, 2006). An additional, only recently detected, outstanding group of addiction systems is represented by the operator repressor titration system (Cranenburgh et al., 2001).

However, none of the mentioned systems gave 100% plasmid stability. An example is the pTF-FC2 system, which resulted only in a three-fold—but not complete stabilization of the target plasmid (Smith and Rawlings, 1997). One reason might be the fact that randomly occurred point mutations result in a drastically reduced binding or general activity of the toxin/antitoxin. On the other hand, the plasmid-mediated toxin/antitoxin systems had the general problem of randomly loosing the plasmid, and the remaining cells did not inevitably die because of the diluted and reduced toxin or antitoxin concentration. In some cases the used host bacterium is RecA positive, which can lead to spontaneous recombinations of the genes involved during cultivation. To avoid this problem, we chose the RecA-negative strain *E. coli* HMS174(DE3). A general critical aspect of catabolic addiction systems is the restriction to mineral salts media with a defined carbon source to enable such systems to function properly. Additionally, catabolic pathways could be (partially) bypassed. For instance the use of other available carbon sources in the culture broth (e.g. carbon dioxide in chemolithoautotrophic organisms) is possible and cannot be completely excluded. In contrast, our anabolism-based system exhibits no other loop way to bypass the focusing pathway. Outstanding for this system the cultivation is not addicted to a defined carbon source and can be used with complex media. The use of complex media is an important aspect in industry because of of the use of cheap agricultural commodities such as corn steep liquor, a co-product from the wet milling of maize.

Surprisingly, the addiction system constructed in this study seems to be the first example of an anabolism-based addiction system among the metabolism-based addiction systems. Since we could not detect any plasmid loss (Figs. 4 and 5), reduced enzyme activity or diminished cyanophycin accumulation (Table 3), this addiction system confers high plasmid stability to *E. coli*. Theoretically, anabolism-based systems have several advantages in comparison to catabolism-based systems because of the possible problems described above. In this study, cyanophycin production was applied as an example to demonstrate the functionality of the generated addiction system. Growth experiments indicated that strains harboring genes coding for the MVA pathway, including the *ispH* knockout mutant harboring the plasmid that encodes the MVA pathway, exhibited slightly weaker growth in comparison to the control strain. This might be due to the additional efforts of the cells to synthesize now also the necessary enzymes for the MVA pathway. Furthermore, the metabolite flow in these recombinant strains needs probably to be optimized and adjusted. Such an optimization was not in the scope of the present study, but would be useful for further applications.

Additionally we demonstrated the *in vivo* functionality of the applied artificial mevalonate pathway. To our knowledge, this study presents the first proof of functionally active 5-MVPP-decarboxylase (EC. 4.1.1.33) from *S. aureus* in *E. coli*.

The knockout mutant synthesized less cyanophycin than the control strain (Table 3); an explanation might be that this mutant is dependent on the non-regulated essential IPP supply and the

putative accumulation of intermediates of the disrupted DXP pathway. These circumstances are absent in the control strain. We want to emphasize that only the strains with pCOLADuet-1 plasmid background, with or without *ispH* knockout, can be compared directly regarding growth and plasmid stability due to the divergent replicons of pET23a and pCOLADuet. During light microscopic investigations we observed spheroplast-like cell morphologies (data not shown). Similar observations were made before in the context of demonstrating the essential character of *ispH* (McAteer et al., 2001).

Due to its outstanding stability, the addiction system described in this study exhibits a multitude of putative applications for biotechnology. In this study, we exemplarily described a one-step biosynthesis pathway to produce the polymer cyanophycin in a recombinant strain. However, biosynthesis pathways, which are constituted of several steps, can be of course also applied instead (for example, biosynthesis of polyhydroxybutyrate, biofuels or isoprenoids). Furthermore, the described system could additionally be also applied in the industrial production of pharmaceutical proteins or chemicals like insulin, somatostatin, growth hormones or the anti-malaria drug precursor amorpha-4,11-diene. In addition, this or related addiction systems could be applied in a variety of other microorganisms. For example, the system could be also applied in industrially relevant eukaryotic microorganisms (i.e. *Pichia pastoris*, *Saccharomyces cerevisiae*) naturally harboring the MVA pathway; in this case a gene essentially involved in the MVA pathway should be deleted, after the introduction of an artificial episomally encoded DXP pathway.

Acknowledgments

We thank Ursula Malkus (Institut für Medizinische Physik und Biophysik) for expert preparation of the electron microscopic specimen. Provision of the plasmid pCOLADuet1::MVA1–5 by Dr. Johannes Kaiser (EVONIK Industries AG) is gratefully acknowledged. This project was financially supported by EVONIK Industries AG, and the federal state North-Rhine Westphalia co-financed by the European Union.

References

Aboulmagd, E., Oppermann-Sanio, F.B., Steinbüchel, A., 2000. Molecular characterization of the cyanophycin synthetase from *Synechocystis* sp. strain PCC 6308. Arch. Microbiol. 174, 297–306.

Aboulmagd, E., Voss, I., Oppermann-Sanio, F.B., Steinbüchel, A., 2001. Heterologous expression of cyanophycin synthetase and cyanophycin synthesis in the industrial relevant bacteria *Corynebacterium glutamicum* and *Ralstonia eutropha* and in *Pseudomonas putida*. Biomacromolecules 2, 1338–1342.

Bochar, D.A., Tabernero, L., Stauffacher, C.V., Rodwell, V.W., 1999. Aminoethylcysteine can replace the function of the essential active site lysine of *Pseudomonas mevalonii* 3-hydroxy-3-methylglutaryl coenzyme A reductase. Biochemistry 38, 8879–8883.

Boucher, Y., Doolittle, W.F., 2000. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. Mol. Microbiol. 37, 703–716.

Connolly, D.M., Winkler, M.E., 1989. Genetic and physiological relationships among the *miaA* gene, 2-methylthio-*N*6-(delta 2-isopentenyl)-adenosine tRNA modification, and spontaneous mutagenesis in *Escherichia coli* K-12. J. Bacteriol. 171, 3233–3246.

Cranenburgh, R.M., Hanak, J.A., Williams, S.G., Sherratt, D.J., 2001. *Escherichia coli* strains that allow antibiotic-free plasmid selection and maintenance by repressor titration. Nucleic Acids Res. 29, E26.

Elbahloul, Y., Krehenbrink, M., Reichelt, R., Steinbüchel, A., 2005. Physiological conditions conducive to high cyanophycin content in biomass of *Acinetobacter calcoaceticus* strain ADP1. Appl. Environ. Microbiol. 71, 858–866.

Elbahloul, Y., Steinbüchel, A., 2006. Engineering the genotype of *Acinetobacter* sp. strain ADP1 to enhance biosynthesis of cyanophycin. Appl. Environ. Microbiol. 72, 1410–1419.

Frey, K.M., Oppermann-Sanio, F.B., Schmidt, H., Steinbüchel, A., 2002. Technical-scale production of cyanophycin with recombinant strains of *Escherichia coli*. Appl. Environ. Microbiol. 68, 3377–3384.

Gerdes, K., Christensen, S.K., Løbner-Olesen, A., 2005. Prokaryotic toxin–antitoxin stress response loci. Nat. Rev. Microbiol. 3, 371–382.

Hai, T., Frey, K.M., Steinbüchel, A., 2006. Activation of cyanophycin synthetase of *Nostoc ellipsosporum* strain NE1 by truncation at the carboxy-terminal region. Appl. Microbiol. Biotechnol. 72, 7652–7660.

Hartree, E.F., 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48, 422–427.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R., 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61–68.

Jensen, R.B., Gerdes, K., 1995. Programmed cell death in bacteria: protein plasmid stabilization systems. Mol. Microbiol. 17, 205–210.

Krehenbrink, M., Oppermann-Sanio, F.B., Steinbüchel, A., 2002. Evaluation of non-cyanobacterial genome sequences for occurrence of genes encoding proteins homologous to cyanophycin synthetase and cloning of an active cyanophycin synthetase from *Acinetobacter* sp. strain DSM 587. Arch. Microbiol. 177, 371–380.

Kuzuyama, T., 2002. Mevalonate and non-mevalonate pathways for the biosynthesis of isoprene units. Biosci. Biotechnol. Biochem. 66, 1619–1627.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

McAteer, S., Coulson, A., McLennan, N., Masters, M., 2001. The *lytB* gene of *Escherichia coli* is essential and specifies a product needed for isoprenoid biosynthesis. J. Bacteriol. 183, 7403–7407.

Neumann, K., Stephan, D.P., Ziegler, K., Hühns, M., Broer, I., Lockau, W., Pistorius, E.K., 2005. Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants. Plant Biotechnol. J. 3, 249–258.

Ogura, T., Hiraga, S., 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA 80, 4784–4788.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17, 208–212.

Sallam, A., Steinbüchel, A., 2008. Anaerobic and aerobic degradation of cyanophycin by the denitrifying bacterium *Pseudomonas alcaligenes* strain DIP1 and role of three other coisolates in a mixed bacterial consortium. Appl. Environ. Microbiol. 74, 3434–3443.

Sanders, J., Scott, E., Weusthuis, R., Mooibroek, H., 2007. Bio-Refinery as the bio-inspired process to bulk chemicals. Macromol. Biosci. 7, 105–117.

Schwamborn, M., 1998. Chemical synthesis of polyaspartates: a biodegradable alternative to currently used polycarboxylate homo- and copolymers. Polym. Degrad. Stabil. 59, 39–45.

Scott, E., Peter, F., Sanders, J., 2007. Biomass in the manufacture of industrial products—the use of proteins and amino acids. Appl. Microbiol. Biotechnol. 75, 751–762.

Simon, R.D., Weathers, P., 1976. Determination of the structure of the novel polypeptide containing aspartic acid and arginine which is found in cyanobacteria. Biochim. Biophys. Acta 420, 165–176.

Smith, A.S., Rawlings, D.E., 1997. The poison-antidote stability system of the broad-hostrange *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol Microbiol. 26 (5), 961–970.

Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31–43.

Schweder, T., Schmidt, I., Herrmann, H., Neubauer, P., Hecker, M., Hofmann, K., 1992. An expression vector system providing plasmid stability and conditional suicide of plasmid-containing cells. Appl. Microbiol. Biotechnol. 38, 91–93.

Steinle, A., Oppermann-Sanio, F.B., Reichelt, R., Steinbüchel, A., 2008. Synthesis and accumulation of cyanophycin in transgenic strains of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 74, 3410–3418.

Voss, I., Steinbüchel, A., 2006. Application of a KDPG-aldolase gene-dependent addiction system for enhanced production of cyanophycin in *Ralstonia eutropha* strain H16. Metabol. Eng. 8, 66–78.

Zielenkiewicz, U., Cegłowski, P., 2001. Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems. Acta Biochim. Pol. 48, 1003–1023.