Available online at www.sciencedirect.com
SCIENCE DIRECT®
Metabolic Engineering 8 (2006) 66–78
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Application of a KDPG-aldolase gene-dependent addiction system for enhanced production of cyanophycin in *Ralstonia eutropha* strain H16

Ingo Voss, Alexander Steinbüchel*

Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Corrensstraße 3, D-48149 Münster, Germany

Received 23 March 2005; received in revised form 22 August 2005; accepted 6 September 2005
Available online 2 November 2005

Abstract

Two different recombinant plasmids both containing the cyanophycin synthetase gene (*cphA*) of *Synechocystis* sp. strain PCC6308 but differing concerning the resistance marker gene were tested for their suitability to produce high amounts of cyanophycin in recombinant strains of *Ralstonia eutropha*. Various cultivation experiments at the 30-L scale revealed very low cyanophycin contents of the cells ranging from 4.6% to 6.2% (w/w) of cellular dry weight (CDW) only, most probably because most cells had lost the corresponding plasmid during cultivation. To establish a cost effective and high efficient system for production of cyanophycin at larger scales using recombinant strains of *R. eutropha*, we applied two strategies: First, we integrated *cphA* into the dispensable chromosomal L-lactate dehydrogenase gene (*ldh*) of *R. eutropha*. Depending on the cultivation conditions used, relatively low cyanophycin contents between 2.2% and 7.7% (w/w) of CDW were reproducibly detected, which might be due to weak expression or low gene dosage in the single *cphA* copy strain of *R. eutropha*. In a second strategy we constructed a KDPG-aldolase gene (*eda*)-dependent addiction system, which combined features of a multi-copy plasmid with stabilized expression of *cphA*. Flasks experiments revealed that the cells accumulated extraordinarily high amounts of cyanophycin between 26.9% and 40.0% (w/w) of CDW even under cultivation conditions lacking cyanophycin precursor substrates or plasmid stabilizing antibiotics. Cyanophycin contents of up to 40.0% (w/w) of CDW were also obtained at a 30-L scale or a 500-L pilot-plant scale under such non-selective conditions. This demonstrates impressively that the stabilizing effect of the constructed *eda*-dependent addiction system can be used for production of enhanced amounts of cyanophycin at a larger scale in recombinant strains of *R. eutropha*.

Keywords: Addiction system; *cphA*; Cyanophycin; Cyanophycin synthetase; Genomic integration; KDPG-aldolase; Metabolic engineering; Plasmid stability; *Ralstonia eutropha*

1. Introduction

Cyanophycin [multi-L-arginyl-poly(L-aspartic acid)] is a protein-like polymer, which is common to cyanobacteria and was discovered more than 100 years ago (Borzi, 1887). This polymer contains aspartic acid and arginine at an about equimolar ratio, and is arranged as a polyaspartic acid backbone with arginine moieties linked to the β-carboxyl group of each aspartate by its α-amino group (Simon, 1976). Cyanophycin serves as a temporary nitrogen, carbon and energy reserve and usually accumulates as cytoplasmic inclusions during the transition from the exponential to the stationary growth phase (Mackerras et al., 1990). At neutral pH and physiological ionic strength cyanophycin is insoluble. Cyanophycin is of biotechnological interest because a derivative with reduced arginine content can be obtained by partial chemical hydrolysis (Joentgen et al., 1998), which can be used as a biodegradable substitute for polyacrylate in various technical applications (Schwamborn, 1998).

Intracellular synthesis and degradation of cyanophycin are catalyzed by cyanophycin synthetase, which is encoded by cyanophycin synthetase gene (*cphA*) (Ziegler et al., 1998), and cyanophycinase, which is encoded by *cphB* (Richter et al., 1999), respectively. The polymerization

*Corresponding author. Fax: +49 251 8338388.
E-mail address: steinbu@uni-muenster.de (A. Steinbüchel).

1096-7176/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2005.09.003

reaction yields polydisperse cyanophycin with molecular weights ranging from 25,000 to 100,000 Da (Simon, 1976). The activity of cyanophycin synthetase depends on the presence of L-aspartic acid, L-arginine, ATP, Mg^{2+}, K^{+}, a sulfhydryl compound and cyanophycin as a primer (Simon, 1976; Ziegler et al., 1998; Aboulmagd et al., 2000). Cyanophycin synthetase has been purified from various cyanobacteria, like *Anabaena variabilis* (Ziegler et al., 1998), *Synechocystis* sp. strain PCC6308 (Aboulmagd et al., 2001a) and the thermophilic *Synechococcus* sp. strain MA19 (Hai et al., 1999). *CphA* was cloned from *A. variabilis* (Ziegler et al., 1998), *Synechocystis* sp. strains PCC6803 (Ziegler et al., 1998; Oppermann-Sanio et al., 1999) and PCC6308 (Aboulmagd et al., 2000), *Synechococcus* sp. strain MA19 (Hai et al., 2002), and *Thermosynechococcus elongatus* (Berg et al., 2000).

More recently, *cphA* homologous genes were also found in several chemotrophic bacteria, like *Acinetobacter* sp. strain ADP1, *Bordetella* sp., *Clostridium botulinum*, *Desulfitobacterium hafniense*, *Nitrosomonas europaea* and others (Krehenbrink et al., 2002; Ziegler et al., 2002). Heterologous expression of *cphA* from *Acinetobacter* sp. strain ADP1 and *Desulfitobacterium hafniense* in *Escherichia coli* resulted in detectable CphA activity and also cyanophycin accumulation, indicating that CphAs of both organisms were functionally active (Krehenbrink et al., 2002; Ziegler et al., 2002). In addition, cyanophycin accumulation occurs also in the wild type *Acinetobacter* sp. strain ADP1 under phosphate limited culture conditions (Krehenbrink et al., 2002; Elbahloul et al., 2005).

For technical production of cyanophycin, heterologous expression of different cyanobacterial *cphA* genes was demonstrated in recombinant strains of *E. coli* (Frey et al., 2002), *Ralstonia eutropha*, *Corynebacterium glutamicum* and *Pseudomonas putida* (Aboulmagd et al., 2001b). More recently it was shown that production of cyanophycin in recombinant strains of *R. eutropha* and *P. putida* is significantly influenced by the source of *cphA* gene, the accumulation of another storage compound like polyhydroxyalkanoates (PHA) as well as by the concentration of precursor substrates (Voss et al., 2004). Very recently *cphA* from *T. elongatus* strain BP-1 was successfully transferred and expressed in tobacco and potato plants (Neumann et al., 2005).

In this study, we report on further strain optimization for cyanophycin production with recombinant *R. eutropha* using stabilized expression systems for *cphA*, which minimize plasmid instability especially during cultivation at a 30- and 500-L scale. A high rate of plasmid instability is a well known problem of recombinant high-level protein expression and is often correlated with a strong decrease in productivity. To overcome this problem and to establish a stable process for cyanophycin production without the need to add antibiotics, we constructed *R. eutropha* strains either containing a single copy of *cphA* integrated into the genome or using a KDPG-aldolase gene-dependent addiction system.

2. Material and methods

2.1. Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C in Luria Bertani (LB) medium (Sambrook et al., 1989). *R. eutropha* was grown either in Nutrient Broth (NB) medium (Difco Laboratories, Detroit, USA) or in mineral salts medium (MSM) according to Schlegel et al. (1961) at a temperature of 30 °C. Sodium gluconate, fructose or sodium lactate were used as carbon sources at concentrations of 1–2% (w/v), and aspartic acid and arginine were added as supplements at concentrations of 0.2% (w/v) in some experiments if indicated in the text. For selection of plasmid carrying strains, antibiotics were added to the media at the following concentrations (μg/ml): ampicillin (75, *E. coli*), kanamycin (50, *E. coli* and 300, *R. eutropha*) and tetracycline (12.5, *E. coli* and 25, *R. eutropha*).

2.2. Preparation of cell extracts

After entering the stationary growth phase, cells were harvested by centrifugation (10 min, 2800*g*, 4 °C), washed once with 50 mM Tris–HCl buffer (pH 8.2) and resuspended with 2 mL buffer per gram of fresh cell mass. Cells of *E. coli* and *R. eutropha* were disintegrated by a three-fold passage through an ice-cooled French press cell at 96 MPa. The supernatants of high-speed centrifugations of the broken cells (1 h, 100,000*g*, 4 °C) were desalted on NAP5 columns (Pharmacia Biotech, Freiburg, Germany) and served as 'soluble cell fractions' for enzyme activity measurements.

2.3. Analysis of ammonium and gluconate

The concentration of ammonium was estimated in cell-free supernatants employing ammonium test bars (Merck, Darmstadt, Germany) or a gas sensitive ammonium electrode (Type 152303000; Mettler Toledo GmbH, Greifensee, Switzerland).

The concentration of gluconate was estimated in cell-free supernatants employing an enzyme test kit for D-gluconic acid (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany) following the instructions of the manufacturer.

2.4. Enzyme assays

The activity of KDPG-aldolase (EC 4.1.2.14) was determined by a spectrophotometric assay in 45 mM triethanolamine hydrochloride (pH 7.6) containing 0.25 mM NADH, 1 mM 2-keto-3-deoxy-6-phosphogluconate (KDPG) and 20 U of L-(+)-lactate dehydrogenase per ml (Blackkolb and Schlegel, 1968).

Cyanophycin synthetase activity was determined by a radiometric assay according to the protocol described previously (Aboulmagd et al., 2000). The activity was

Table 1
Bacterial strains and plasmids used in this study

Bacterial strains or plasmids	Relevant characteristics	Reference or source
Bacterial strain		
Ralstonia eutropha		
H16	Wild type, autotrophic, prototrophic	DSM 428
H16-PHB$^-$4	Mutant of H16 defective in synthesis of PHB	DSM 541
Δ*ldh*/Ωkm-*cphA*	Mutant of H16-PHB$^-$4 with *cphA* of *Synechocystis* sp. PCC6308 integrated into the *ldh* gene	This study
Δ*eda*	Marker-free mutant of H16-PHB$^-$4 with deleted *eda* gene	This study
Escherichia coli		
S17-1	*recA proA thi-1 hsdR*, harbors the RP4 *tra* genes in the chromosome	Simon et al. (1983)
TOP10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (Carlsbad, USA)
Plasmid		
pBluescript SK$^-$	Amp^r, *lacPOZ*, T7 and T3 promoter	Stratagene (San Diego, CA)
pBBR1MCS-2	Km^r, broad host range vector, *lacPOZ*′	Kovach et al. (1995)
pBBR1MCS-3	Tc^r, broad host range vector, *lacPOZ*′	Kovach et al. (1995)
pSKsymΩkm	Ωkm in pSKsym	Overhage et al. (1999)
pEX100T	Suicide vector, km^r, *lacPOZ*‘, *oriT*, *sacB*	Schweizer and Hoang (1995)
pJQmp18-Tc^r	Suicide vector, Tc^r, *sacB*, oriV, oriT, *traJ*	Pötter et al. (2005)
pBBR1MCS-2::*cphA*	pBBR1MCS-2 harboring *cphA* from *Synechocystis* sp. strain PCC6308	Aboulmagd et al. (2001a, b)
pBBR1MCS-3::*cphA*	pBBR1MCS-3 harboring *cphA* from *Synechocystis* sp. strain PCC6308	This study
pSK$^-$::*cphA*	3.3-kbp PCR product from *Synechocystis* sp. strain PCC6308 DNA in pBluescript SK$^-$ harboring *cphA* colinear to *lacPOZ*′	Aboulmagd et al. (2000)
pSK$^-$::Ωkm-*cphA*	pBlueskript SK$^-$ harboring 1.1-kbp Ωkm and 3.3-kbp *cphA* from *Synechocystis* sp. PCC6308	This study
pEX100T::*ldh*	pEX100T harboring 1.7-kbp fragment containing *ldh* gene	This study
pEX100T::Δ*ldh*/Ωkm-*cphA*	pEX100T harboring *ldh* gene interrupted by 4.3-kbp Ωkm-*cphA* fragment	This study
pSK$^-$::*eda*	pBlueskript SK$^-$ with a 1.63-kbp *Pst*I–*Xba*I PCR product from *R. eutropha* H16 genomic DNA containing *eda* gene	This study
pSK$^-$::Δ*eda*	pBlueskript SK$^-$ harboring a 1-kbp fragment containing disrupted *eda* gene	This study
pJQmp18-Tc^r::Δ*eda*	pJQmp18-Tc^r with 1-kbp disrupted *eda* gene	This study
pBBR1MCS-2::*cphA*/*eda*	pBBR1MCS-2 harboring 3.3-kbp *cphA* and 0.64-kbp *eda* gene of *R. eutropha* H16	This study

Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; *eda*, 2-keto-3-desoxy-6-phosphogluconate aldolase gene; *ldh*, lactate dehydrogenase gene; PCC, Pasteur Culture Collection of Cyanobacteria, Paris, France; Km, kanamycin.

measured by following the incorporation of (U-^{14}C) L-arginine into insoluble cyanophycin.

Enzyme activities were measured at 30 °C. One unit of enzyme activity was defined as the transformation of 1 μmol of substrate per min. Specific activity is given in units per milligram of protein.

2.5. DNA isolation and manipulation

Total genomic DNA of *R. eutropha* was isolated according to a procedure described by Marmur (1961). Plasmid DNA was isolated from *E. coli* and *R. eutropha* by the alkaline extraction procedure (Birnboim and Doly, 1979). DNA was digested with various restriction endonucleases under the conditions described by Sambrook et al. (1989) or by the manufacturer. *Pfx*-DNA polymerase (Invitrogen, Karlsruhe, Germany) was used according to the instructions of the manufacturer. Primers were purchased from MWG-Biotech AG (Ebersberg, Germany). Other DNA-manipulating enzymes were used as described by the manufacturers. DNA fragments were isolated from agarose gels by using the NucleoTrap kit (Machery and Nagel, Düren, Germany).

2.6. Transfer of plasmids

Plasmids were transferred into *E. coli* by employing the $CaCl_2$ method (Sambrook et al., 1989), whereas the transfer of plasmids into *R. eutropha* was performed by conjugation using *E. coli* S17-1 as donor strain (Friedrich et al., 1981).

2.7. Cloning of cphA into the broad host range vector pBBR1MCS-3

The cyanophycin synthetase gene of *Synechocystis* sp. strain PCC6308 was amplified by PCR using plasmid DNA of the previously constructed hybrid plasmid pBBR1MCS-2::*cphA* (Aboulmagd et al., 2001b) and oligonucleotides #1 and #2 (Table 2). After digestion with *Xho*I–*Xba*I, the fragment was cloned into the broad host range vector pBBR1MCS-3, which was digested with the

Table 2
Oligonucleotides used in this study

No. #	Name	Sequence (5′→3′)
1	up-*cphA* (*Xho*I)	AAAAAC|TCGAGATGGGGCATCACATGATCGCCGG
2	down-*cphA* (*Xba*I)	AAAAAT|CTAGATACTCACTATTCACTACTGAGATG
3	up-*ldh* (*Eco*RI)	AAAG|AATTCCATCCCCGTGACGATCATCGGCTA
4	down-*ldh* (*Eco*RI)	AAAG|AATTCCGGATTATTAGCCGACGGCGCCTTC
5	up-Ωkm (*Sal*I)	AAAG|TCGACACAGCAAGCGAACCGGAATGACCAGCTGGGG
6	down-*cphA* (*Sal*I)	AAAG|TCGACTACTCACTATTCACTACTGAGATG
7	up-eda_{H16} (*Xba*I)	AAAAAT|CTAGAGTCACGCTGGACACCAACCTGC
8	down-eda_{H16} (*Pst*I)	AAAAACTGCA|GATTCCCTGCGACCTGGCTGC
9	Inv1-eda_{H16}	TCAGGGAAGCAGGAGTTCAGGC
10	Inv2-eda_{H16}	GAGAAACGGCACGCCATGC
11	up-eda_{H16} (*Xba*I)	AAAAAT|CTAGACTGATCCCTACTCCGAGACAGCCATGC
12	down-eda_{H16} (*Sac*I)	AAAAAGAGCT|CTTGCGCTGCCGCTGCTCA
13	up-*ldh*/Ωkm	TCGATACCGCCACCGTGACC
14	down-*ldh*/Ωkm	GCCGATTGTCTGTTGTGCCC
15	up-*cphA*/*ldh*	ACGCAGTTTTAACCCTGGGGC
16	down-*cphA*/*ldh*	TCAGGCGACGGCGTTATCG

same restriction endonucleases. After transformation in *E. coli* S17-1, the resulting plasmid pBBR1MCS-3::*cphA* was transferred by conjugation into *R. eutropha* H16-PHB^{-}4.

2.8. Analysis of plasmid stability

For plasmid stability tests, sterile samples were withdrawn from the fermenter at the beginning and at the end of a cultivation. Hundred microliters of these samples were appropriately diluted and spread onto MSM agar plates containing no antibiotic or containing kanamycin or tetracycline depending on the plasmid used. The plates were incubated for 24 h at 30 °C, and the colony forming units (CFU) were counted. The values obtained for MSM agar plates without antibiotics were set as 100%, and the % CFU values obtained in MSM plus kanamycin or tetracycline were related to the latter.

2.9. Genomic integration of cphA

For integration of the *cphA* gene of *Synechocystis* sp. strain PCC6308 into the genome of *R. eutropha* H16-PHB^{-}4, the L-lactate dehydrogenase (EC 1.1.1.27) encoding *ldh* gene (Jendrossek et al., 1993) was chosen as appropriate target sequence (Fig. 1A), and the previously constructed plasmid pBluescript SK^{-} harboring *cphA* of *Synechocystis* sp. PCC6308 (Aboulmagd et al., 2000) was used. The kanamycin resistance gene was isolated from *Sma*I-digested pSKsymΩkm (Overhage et al., 1999) and was then subcloned into *Sma*I digested pSK^{-}::*cphA* yielding pSK^{-}::Ωkm-*cphA*. The *ldh* gene was amplified from genomic DNA of strain H16 using oligonucleotides #3 and #4. The obtained PCR product was digested with *Eco*RI and subsequently ligated into *Eco*RI-digested suicide vector pEX100T (Schweizer and Hoang, 1995). The Ωkm-*cphA* fragment was amplified by PCR using oligonucleotides #5 and #6 and was then inserted into the central part of the *ldh* encoding region after *Sal*I digestion of both constructs. The resulting plasmid pEX100T::Δ*ldh*/Ωkm-*cphA* was transformed into *E. coli* Top10 to analyze the specific activity of cyanophycin synthetase. To integrate the Ωkm-*cphA* fragment into the *ldh* structural gene of *R. eutropha* H16-PHB^{-}4 by homologous recombination, the suicide plasmid was transformed into *E. coli* S17-1, which served as donor strain in conjugative transfer.

2.10. Disruption of the KDPG-aldolase encoding eda gene

A 1.63-kbp DNA region starting 533-bp upstream and ending 450-bp downstream of the *eda* gene was amplified by PCR from genomic DNA of *R. eutropha* H16 using oligonucleotides #7 and #8 (Table 2 and Fig. 2). After *Xba*I–*Pst*I digestion, the fragment was cloned into pBlueskript SK^{-} resulting in pSK^{-}::*eda*. Inverse PCR was done to amplify the regions up- and downstream of *eda* using oligonucleotides #9 and #10, whereby the coding region of *eda* was completely excised. After ligation of the hybrid plasmid, the Δ*eda* fragment was isolated after *Xba*I–*Pst*I digestion and subcloned into the suicide vector pJQmp18-Tc^{r} (Pötter et al., 2005). The resulting plasmid was used for homologous recombination in *R. eutropha* H16-PHB^{-}4 after transformation of *E. coli* S17-1 and conjugative transfer of the plasmid to *R. eutropha*.

For construction of a KDPG-aldolase gene-deficient addiction system, which combines the complementation of the KDPG-deficient mutant of *R. eutropha* H16-PHB^{-}4 with expression of *cphA*, the *eda* gene was amplified from total genomic DNA of *R. eutropha* H16 by PCR using oligonucleotides #11 and #12. The resulting 678-bp fragment, which comprised the complete coding region of the *eda* gene and also its Shine–Dalgarno sequence, was digested with *Xba*I-*Sac*I and cloned into the above mentioned hybrid plasmid pBBR1MCS-2::*cphA* directly downstream of the *cphA* gene (Fig. 3). The resulting plasmid was transferred into *R. eutropha* H16-PHB^{-}4-Δ*eda* via *E. coli* S17-1 by conjugation.

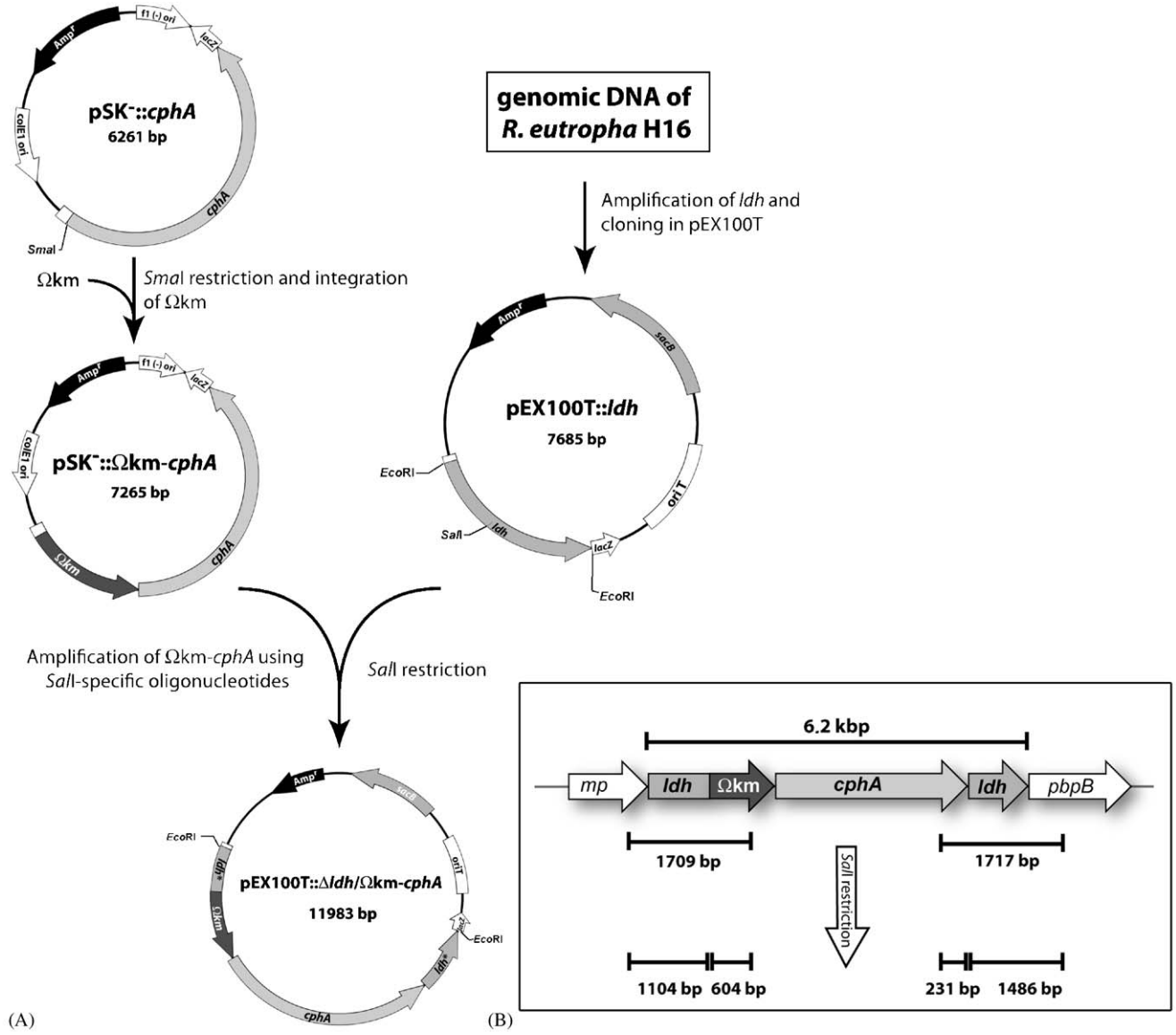


Fig. 1. Construction of the suicide plasmid pEX100T::*ldh*/ΩKm-*cphA* to integrate a single-copy of *cphA* from *Synechocystis* sp. PCC6308 into the *ldh* structural gene of *R. eutropha* H16 (A) and verification of the *cphA* integration mutant *R. eutropha* H16-PHB⁻4-Δ*ldh*/ΩKm-*cphA* by PCR (B). For amplification of a 1709-bp fragment (5′-integration side) oligonucleotides #13 and #14 were used, whereas for amplification of a 1717-bp fragment (3′-integration side) oligonucleotides #15 and #16 were used (Table 2). The resulting PCR products were digested with *Sal*I to ensure chromosomal integration of the constructed ΩKm-*cphA* cassette.

2.11. 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.

2.12. Purification and determination of cyanophycin

Cyanophycin was isolated according to the method described by Simon and Weathers (1976) or by the acid extraction procedure described by Frey et al. (2002). The amino acid constituents of cyanophycin were determined by HPLC as described previously (Aboulmagd et al., 2000).

2.13. 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 cyanophycin was done with Serva Blue R. Protein concentrations were determined by the procedure of Bradford (1976).

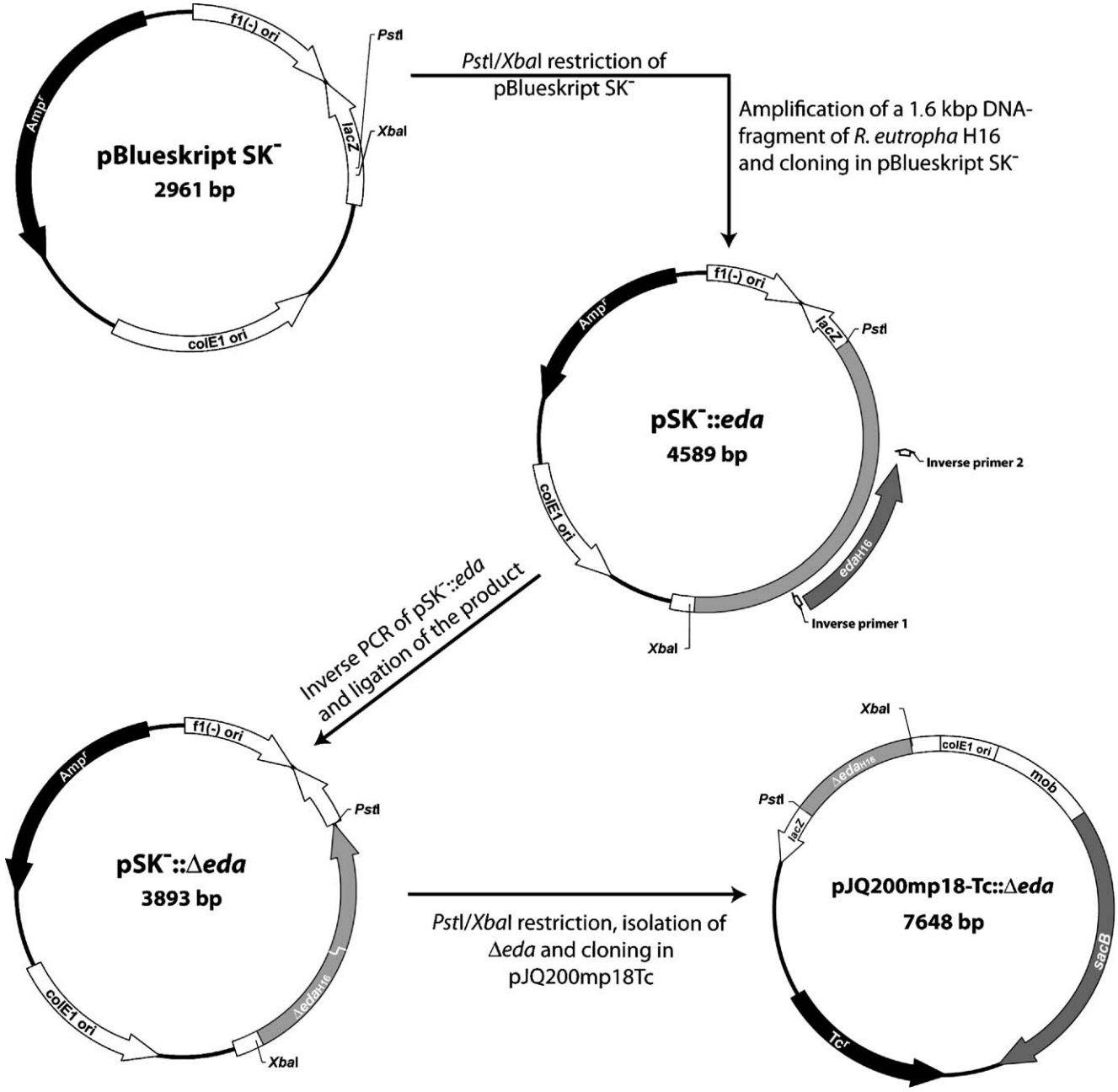


Fig. 2. Construction of the suicide plasmid pJQmp18-Tc^r::Δ*eda* which was used to completely excise the KDPG-aldolase gene (*eda*) from the genome of *R. eutropha* H16-PHB$^-$4.

2.14. Cultivation at 30- or 500-L scale

Cultivations of recombinant *R. eutropha* strains producing cyanophycin were done at a 30- or 500-L scale using either a Biostat UD30 or Biostat D650 stainless steel reactor (B. Braun Biotech International, Melsungen, Germany) which were described previously (Voss and Steinbüchel, 2001).

Cultivations were done at 30 °C either under unrestricted or restricted oxygen conditions. For unrestricted oxygen conditions stirrer speeds between 100 and 500 rpm and aeration rates of maximum 1.0 vvm were used. Restricted oxygen conditions were performed using constant stirrer speeds of 150 rpm and aeration rates of 0.3 vvm during the whole time course of the cultivation. The pH in the medium was controlled by automated addition of 4 N HCl or NaOH. Foam was preferentially controlled by the mechanical foam destroyer; only if this was not sufficient, chemical antifoam agents such as "Silikon Antischaum Emulsion SLE" (Wacker, Darwin Vertriebs GmbH, Ottobrunn, Germany) were added.

2.15. Cell harvest

Small samples withdrawn for analysis of, e.g. medium components were separated into a cell pellet and a cell free

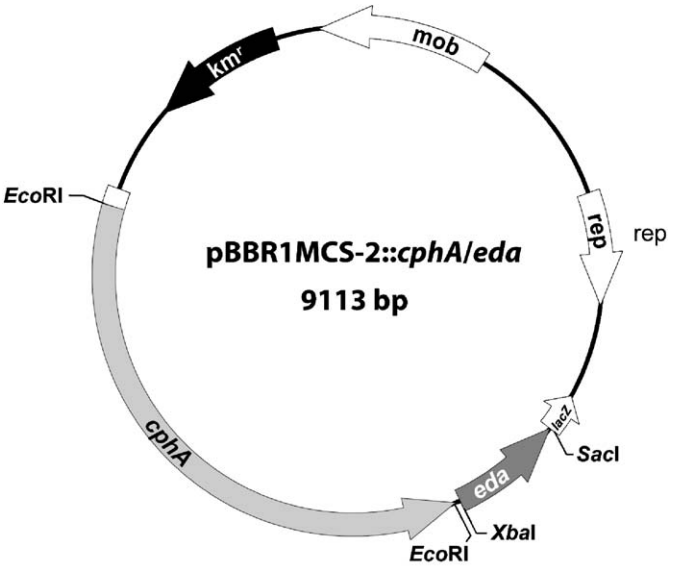


Fig. 3. Map of plasmid pBBR1MCS-2::*cphA*/*eda* used to construct an addiction system for improved production of cyanophycin in *R. eutropha*.

supernatant by centrifugation in various bench top centrifuges. Cells from 30- or 500-L cultures were harvested by centrifugation in a CEPA type Z41 or Z61 continuous centrifuge (Carl Padberg Zentrifugenbau GmbH, Lahr, Germany).

3. Results

3.1. Plasmid-based expression of cyanophycin synthetase in *R. eutropha*

Two different multi-copy plasmids, both harboring *cphA* of *Synechocystis* sp. PCC6308 but differing concerning the resistance marker gene, were transferred by conjugation to *R. eutropha* H16-PHB^{-}4 in order to produce cyanophycin at a larger scale. During various 30-L batch- or fed-batch cultivations in MSM using gluconate as sole carbon source and in some cultivations aspartic acid or arginine as supplements, very low cyanophycin contents between 4.6 and 6.2% (w/w) of cellular dry weight (CDW) were obtained (data not shown). Detailed studies revealed, that 38% or 62% of the cells of the recombinant strain used either harboring plasmid pBBR1CS-3::*cphA* or pBBR1MC-2::*cphA*, respectively, had lost their plasmid during cultivation (data not shown). Therefore, low productivity of cyanophycin during cultivations of recombinant *R. eutropha* strains might be at least partially due to plasmid instability.

3.2. Chromosomal integration of cphA

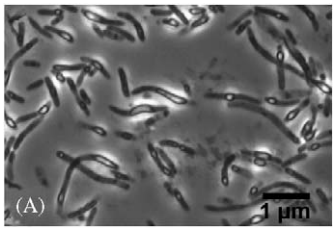
In order to minimize high rates of plasmid loss and to establish a stabilized system for production of cyanophycin without addition of antibiotics, we constructed a mutant of *R. eutropha* H16-PHB^{-}4 containing a single-copy of *cphA* integrated into the chromosomal L-lactate dehydrogenase (EC 1.1.1.27) gene (*ldh*). The dispensable *ldh* gene was chosen as target for integration to reduce formation of lactate especially under restricted oxygen conditions and also to redirect the metabolic flux towards the formation of intermediates supporting biosynthesis of aspartic acid and arginine and therefore of cyanophycin. Construction of a *cphA* integration cassette was done as described in Material and methods and as shown in Fig. 1A. All steps of plasmid construction were tested for expression of *cphA* and synthesis of cyanophycin to ensure activity and functionality of CphA. Thereby, specific CphA activities between 3.2 and 4.9 as well as cyanophycin contents between 15.0% and 21.5% (w/w) of CDW were detected in recombinant strains of *E. coli* (Table 3). The integration vector pEX100T::*ldh*/Ωkm-*cphA* was subsequently transferred to *R. eutropha* H16-PHB^{-}4 by conjugation and as a consequence of homologous recombination *cphA* integration mutants could be selected on MSM agar plates containing kanamycin and sucrose. The integration of *cphA* into *ldh* was confirmed by PCR as shown in Fig. 1B. This *cphA* integration mutant was then evaluated for expression of *cphA* as well as for accumulation of cyanophycin under different cultivation conditions. It was shown, that this recombinant strain of *R. eutropha* produced reproducible amounts of cyanophycin varying between 2.2% and 7.7% (w/w) of CDW depending on the applied cultivation conditions (Table 3). Previous studies revealed highest amounts of cyanophycin of 7.7% (w/w) of CDW and specific CphA activities of 0.2 U per mg of protein during cultivations under limited oxygen conditions when the MSM was supplemented with additional precursor substrates. However, the amounts of cyanophycin accumulated in the cells were still not extraordinarily high, most probably because of the lower gene dosage in the cells in comparison to plasmid-based expression of *cphA*. Light microscopic examinations clearly showed the difference between a plasmid-based expression of *cphA* in a recombinant strain of *E. coli* and in the single *cphA* gene copy strain of *R. eutropha*. The larger number of refractil cyanophycin granules in recombinant cells of *E. coli* harboring plasmid pEX100T::Δ*ldh*/Ωkm-*cphA*, and the only low number of relatively short granules in cells of the *cphA* integration mutant of *R. eutropha* corresponded well with the cyanophycin contents revealed by HPLC analyses (figure insets A and B in Table 3).

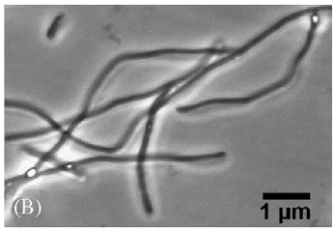
3.3. Construction of a KDPG-aldolase deficient mutant of *R. eutropha*

In another strategy we tried to optimize cyanophycin production with strains of *R. eutropha* by combining the advantages of a multi-copy plasmid with a stabilized expression system for *cphA*. This strategy is based on the structural *eda* gene encoding the KDPG-aldolase (EC 4.1.2.14). This gene is indispensable for growth of *R. eutropha* on gluconate or fructose because the cells

Table 3
Specific CphA activity and synthesis of cyanophycin in recombinant *E. coli* strains harboring *cphA* from *Synechocystis* sp. strain PCC6308 containing plasmids as well as in the *cphA* integration mutant *R. eutropha* H16-PHB^{-}4-Δ*ldh*/Ωkm-*cphA*

Strain/plasmid	Medium	CGP content (% w/w of CDW)	Specific CphA activity [(nmol arginine (min mg protein)$^{-1}$]	Light microscopic photograph
E. coli				
pSK^{-}::*cphA*	LB	20.3±2.0	3.2±0.3	
pSK^{-}::Ωkm-*cphA*	LB	21.5±2.0	4.9±0.3	
pEX100T::Δ*ldh*/Ωkm-*cphA*	LB	15.0±2.0	4.5±0.3	
R. eutropha H16-PHB^{-}4-Δ*ldh*/Ωkm-*cphA*				
With baffles	MSM	2.2±2.0	0.12±0.03	
	MSM + Arg	4.7±2.0	n.d.	
	MSM + Arg/Asp	7.0±2.0	n.d.	
Without baffles	MSM	2.5±2.0	0.2±0.03	
	MSM + Arg	5.2±2.0	n.d	
	MSM + Arg/Asp	7.7±2.0	n.d.	





Abbreviations: Arg, arginine; Asp, aspartic acid; CGP, cyanophycin; n.d., not determined. Light microscopic photographs of (A) recombinant cells of *E. coli* harboring suicide plasmid pEX100T::*ldh*/Ωkm-*cphA* and (B) of the *cphA* integration mutant *R. eutropha* H16-PHB^{-}4-Δ*ldh*/Ωkm-*cphA* cultivated in Erlenmeyer flask without or with baffles and in MSM sometimes supplemented with 0.2% (w/v) arginine and/or aspartic acid as indicated in the table.

catabolize both carbon sources exclusively via the KDPG-pathway (Gottschalk et al., 1964; Schlegel and Gottschalk, 1965; Steinbüchel, 1986). Therefore, we constructed an *eda* deletion mutant as described in Material and methods and shown in Fig. 2. The phenotype of the resulting mutant was investigated in cultivation experiments using various carbon sources as well as by determination of KDPG-aldolase activity in crude extracts. In comparison to the wild type strain H16 or the PHB-negative PHB^{-}4, the *eda* deletion mutant was unable to use gluconate or fructose as sole carbon source for growth, whereas growth with lactate as sole carbon occurred (Fig. 4). In addition, no KDPG-aldolase activity was detectable after growth of the mutant in NB complex medium, whereas the wild type and mutant PHB^{-}4 exhibited KDPG-aldolase activity in the range of 0.1 U per mg of protein (Table 4).

For construction of a stabilized multi-copy *cphA* expression system, the intact *eda* gene of *R. eutropha* H16 was integrated in the above mentioned plasmid pBBR1MCS-2::*cphA* directly downstream of *cphA* from *Synechocystis* sp. strain PCC6308 resulting in plasmid pBBR1MCS-2::*cphA*/*eda* (Fig. 3) which was then transferred into the mutant *R. eutropha* H16-PHB^{-}4-Δ*eda*. By this it was ensured that the recombinant cells could not loose the plasmid harboring *cphA* during growth on, e.g. gluconate as sole carbon source because it also harbored the indispensable *eda* gene. Consequently, the capability to use gluconate as sole carbon source for growth was restored in the recombinant *eda* mutant harboring plasmid pBBR1MCS-2::*cphA*/*eda* (Fig. 4). Furthermore, a nearly two-fold higher specific KDPG-aldolase activity was measured in soluble cell fractions of the recombinant strain, most probably because stronger expression of *eda* localized on a multi-copy plasmid occurred due to a higher gene dosage (Table 4). Since *cphA* is co-expressed with the indispensable *eda* on plasmid pBBR1MCS-2::*cphA*/*eda* in the *eda* mutant of *R. eutropha*, this biological system represents an addiction system ensuring stabilized expression of *cphA* without high rates of plasmid loss.

3.4. Improved production of cyanophycin using the addiction system

The addiction system constructed above offers the possibility of improved production of cyanophycin without addition of plasmid-stabilizing and cost-intensive antibiotics especially at a larger fermentation scale. The applicability of this addiction system to produce high amounts of cyanophycin in *R. eutropha* was investigated in various cultivation experiments. First, flasks experiments were performed to investigate the effect of cultivation conditions and of supplementation of the medium with cyanophycin

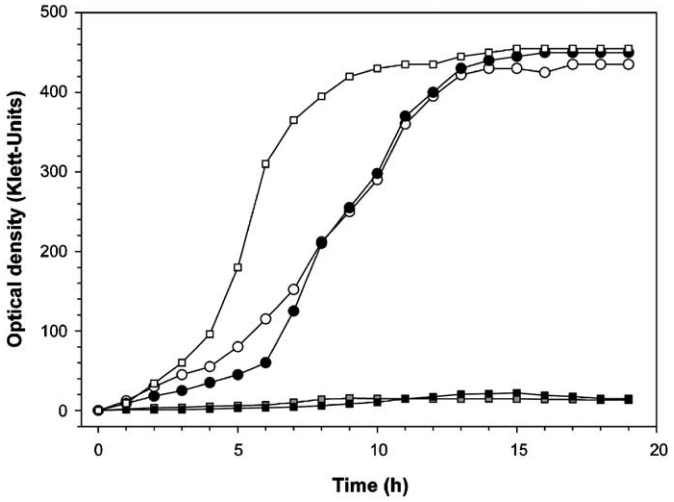


Fig. 4. Growth of *R. eutropha* H16-PHB^{-}4 in comparison to the KDPG-deficient mutant *R. eutropha* H16-PHB^{-}4-Δ*eda* and the complemented mutant *R. eutropha* H16-PHB^{-}4-Δ*eda* (pBBR1MCS-2::*cphA*/*eda*) using different carbon sources. Cells were cultivated at 30 °C and 150 rpm in 300-ml Erlenmeyer flasks containing 150 ml MSM according to Schlegel et al. (1961) and 1.5% (w/v) of the indicated carbon source. The Erlenmeyer flasks were inoculated with 2 ml of an appropriate overnight culture grown in mineral salts medium containing the same carbon source. Growth was recorded in a Klett–Summerson colorimeter equipped with a filter of 520–580 nm. Symbols: (○) *R. eutropha* H16-PHB^{-}4 (pBBR1MCS-2) with gluconate; (▩) *R. eutropha* H16-PHB^{-}4-Δ*eda* with gluconate; (■) *R. eutropha* H16-PHB^{-}4-Δ*eda* with fructose; (□) *R. eutropha* H16-PHB^{-}4-Δ*eda* with lactate and (●) *R. eutropha* H16-PHB^{-}4-Δ*eda* (pBBR1MCS-2::*cphA*/*eda*) with gluconate.

Table 4
Specific enzyme activity of KDPG-aldolase measured in different *R. eutropha* strains

Strain	Medium	Specific KDPG-aldolase activity [(nmol KDPG (min mg protein)$^{-1}$]
R. eutropha H16	MSM	0.092 ± 0.01
R. eutropha H16-PHB^{-}4	MSM	0.102 ± 0.01
R. eutropha H16-PHB^{-}4-Δ*eda*	NB	< 0.010 ± 0.01
R. eutropha H16-PHB^{-}4-Δ*eda* (pBBR1MCS-2::*cphA*/*eda*)	MSM	0.189 ± 0.01

precursor substrates or absence of plasmid-stabilizing antibiotics. The recombinant cells accumulated cyanophycin in an extraordinarily high extent ranging from 26.9% to 40.0% (w/w) of CDW; even in the absence of the amino acids arginine and aspartic acid the cyanophycin contents were as high as 26.9% or 27.7% (w/w) of CDW (Table 5). The cyanophycin contents of cells cultivated in the absence of antibiotics were only marginally lower than of cells cultivated in presence of the respective antibiotic, thus indicating that the plasmid is sufficiently stabilized in the addiction system used (Table 5).

The results obtained in Erlenmeyer flasks encouraged us to use the addiction system for improved cyanophycin production also at large fermentation scale. First, a 30-L fed-batch fermentation was performed under oxygen restricted conditions and without supplementation of antibiotics and cyanophycin precursor substrates (Fig. 5A). After a lag-phase of 6–8 h the exponential growth phase began simultaneously with a drastic decrease in supplied ammonium and dissolved oxygen concentration, the latter of which became limited after 23 h of cultivation. Under these conditions exponential growth turn into linear growth until the end of the fermentation most probably because the oxygen concentration was limited in the culture broth. During this phase, ammonium chloride and gluconate were fed several times as nitrogen and carbon source to prevent exhaustion of these macro-elements which might reduce cell density and accumulation of cyanophycin. At the end of the fermentation after 97 h an optical density ($OD_{600\,nm}$) of 27.5 and a cell density of 6.8 g/L were obtained yielding cells with a cyanophycin content of 35.8% (w/w). Light microscopic examinations revealed that the cells were slightly elongated and that almost each cell contained one or more big cyanophycin granule (Fig. 5B). Analysis of plasmid stability revealed that only 7% of the cells had lost the plasmid during cultivation indicating that the addiction system enabled an improved and stabilized production of cyanophycin with recombinant cells of *R. eutropha* also at larger scale.

A scale up to the 500-L pilot-plant scale confirmed the results obtained at the 30-L scale. A fed-batch fermentation yielded an optical density ($OD_{600\,nm}$) of 42.1 and a cell density of 10.6 g/L with a cyanophycin content of the cells of 32.0% (w/w) of CDW after about 68 h (data not shown).

Table 5
Synthesis and accumulation of cyanophycin by cells of *R. eutropha* H16-PHB^{-}4 (pBBR1MCS-2::*cphA*/*eda*) under different cultivation conditions

Conditions	Medium	CGP content (%, w/w of CDW)	Specific CphA activity [(nmol arginine (min mg protein)$^{-1}$]
With antibiotics	MSM	27.7 ± 2.0	1.9 ± 0.3
	MSM + Arg	31.6 ± 2.0	n.d.
	MSM + Arg/Asp	40.0 ± 2.0	n.d.
Without antibiotics	MSM	26.9 ± 2.0	1.8 ± 0.3
	MSM + Arg	30.8 ± 2.0	n.d.
	MSM + Arg/Asp	38.9 ± 2.0	n.d.

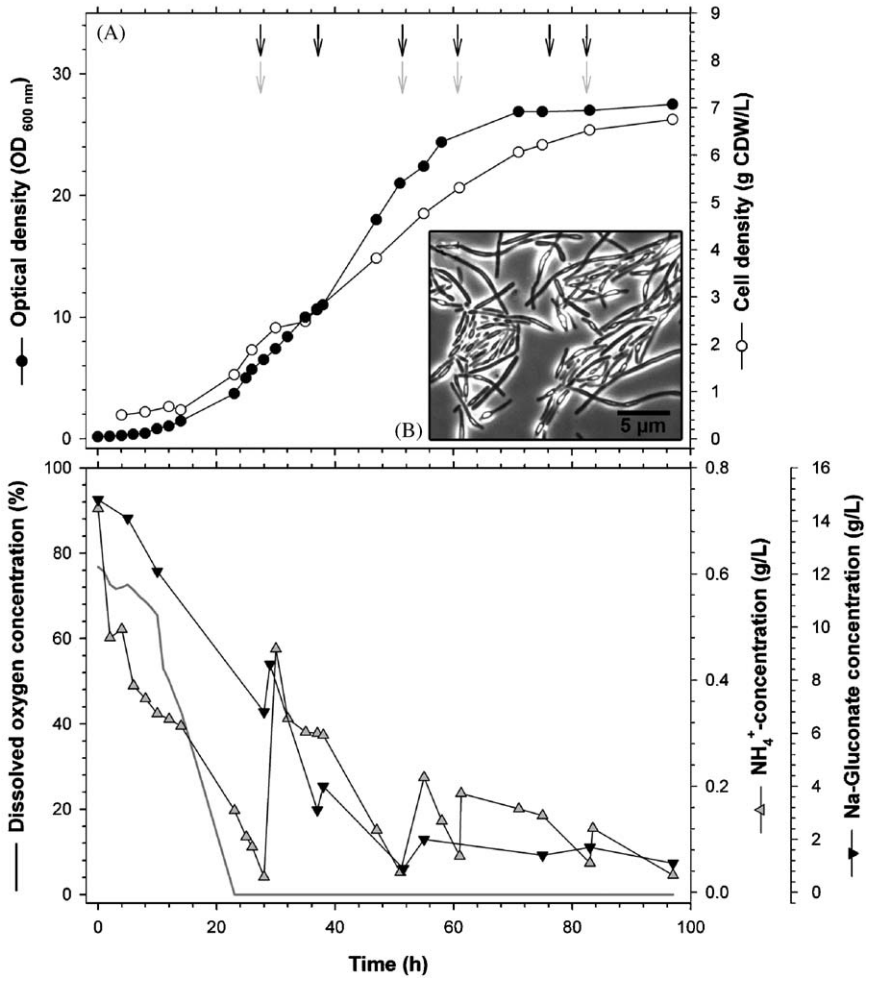


Fig. 5. Fed-batch fermentation of *R. eutropha* H16-PHB^{-}4-Δ*eda* (pBBR1MCS-2::*cphA*/*eda*) under restricted oxygen conditions. (A) The cultivation was done at 30 °C in a 30-L stirred tank reactor (B. Braun, Biotech International, Melsungen, Germany) containing 28 L mineral salts medium with 1.5% (w/v) sodium gluconate as sole carbon source and 0.15% (w/v) NH_4Cl as nitrogen source. Restricted oxygen conditions were performed using constant stirrer speeds of 150 rpm and aeration rates of 0.3 vvm during the whole time course of the cultivation. To prevent limitation of nitrogen or carbon, NH_4Cl (indicated by gray arrows) and gluconate (indicated by black arrows) were fed several times during cultivation. The pH of the medium was controlled at 7.0 by automated addition of 4 M NaOH or 4 M HCl. The concentration of ammonium and gluconate in cell-free supernatants was analyzed as described in Material and methods. Light microscopic photograph of *R. eutropha* H16-PHB^{-}4/Δ*eda* (pBBR1MCS-2::*cphA*/*eda*) cultivated at a 30 L scale (B).

This showed impressively that high plasmid stability allowed the increased production of cyanophycin using recombinant strains of *R. eutropha* even at a large pilot scale.

4. Discussion

A prerequisite to establish expression of a recombinant protein or a functional pathway for production of a recombinant protein or a particular metabolite, respectively, is stable maintenance of the respective gene(s) in the genome of the recombinant production strain. Most often plasmids are used in prokaryotes for this purpose because they can be easily transferred into the host and occur in multiple copies in the cells thereby increasing the amount of the protein to be synthesized and consequently the productivity of the cells. Frequently used multi-copy number plasmids habor antibiotic resistance gene(s) and often require the addition of antibiotics to the medium during cultivation for their stabilization in the cells. This makes the process cost intensive and economically as well as ecologically unfeasible, especially if cultivations are done at a large scale. Moreover, plasmid instability can even occur despite the addition of antibiotics during cultivation, thus also yielding plasmid-free cells and consequently a reduced amount of the product (Zabriskie and Arcuri, 1986).

To overcome the problem of plasmid instability during production of cyanophycin at large scale with recombinant strains of *R. eutropha*, we employed two strategies to stabilize expression of *cphA* of *Synechocystis* sp. strain PCC6308 in *R. eutropha*. One strategy was to integrate *cphA* into one of the two chromosomes (Schwartz and Friedrich, 2001) of *R. eutropha* H16-PHB^{-}4. Genomic integration of a foreign gene is frequently used to overcome

plasmid instability during production of recombinant proteins (Friehs, 2004). It was for example previously shown, that integration of the alcohol dehydrogenase and the pyruvate carboxylase genes of *Zymomonas mobilis* into the *E. coli* chromosome conferred the ability for efficient production of ethanol to *E. coli* (Ohta et al., 1991). In our study we chose the dispensable lactate dehydrogenase gene (*ldh*), because it was known from previous studies that high amounts of lactate are synthesized in *R. eutropha* when the cells are cultivated under conditions of restricted oxygen supply (Steinbüchel et al., 1983). Since limited oxygen supply also promotes synthesis and accumulation of cyanophycin in recombinant strains of *R. eutropha* and *P. putida* (S. Diniz, I. Voss and A. Steinbüchel, unpublished results), disruption of *ldh* by insertion of *cphA* could in addition increase the metabolic flux towards the formation of cyanophycin under these conditions. Although the *cphA* integration mutant of *R. eutropha* accumulated reproducible amounts of cyanophycin under different cultivation conditions, the amounts of cyanophycin were not very high. This was either due to weak expression of *cphA* from the Ωkm- or *ldh*-promoter or due to the lower gene dosage in this single *cphA* gene copy strain in comparison to strains harboring *cphA* on a plasmid. A single copy of an integrated gene will most likely yield lower levels of the recombinant protein than multiple copies of that gene in a multi-copy number plasmid if identical promoters are used. This can be overcome by multiple integrations of the same gene in the genome. It was recently shown that single, double and triple integrations of a recombinant organophosphorylase gene into the chromosome of *R. eutropha* yielded a linear increase in recombinant protein production without instability of the constructs (Srinivasan et al., 2003).

We did not follow up this strategy further because meanwhile an addiction system, which combined features of a multi-copy number plasmid with stabilized expression of *cphA,* was successfully applied and provided much more promising results. The KDPG-aldolase gene-dependent addiction system employed in this study also allowed improved production of cyanophycin with recombinant strains of *R. eutropha* during fermentations at larger scales without addition of antibiotics. In the absence of an antibiotic only 7% of the cells had lost the plasmid even during prolonged fermentation at the 30-L scale. The selective principle, i.e. capture of the plasmid due to its indispensability because the *eda* gene is required for growth on gluconate, caused a 90% increase in plasmid stability in comparison to strains where the selective principle was the presence of an antibiotic. Therefore, the recombinant cells of *R. eutropha* accumulated cyanophycin up to 40% (w/w) of the CDW after cultivation at the 30-L scale and only slightly less after cultivations at the 500-L scale.

Originally, addiction systems were based on the existence of at least two plasmid genes: one gene encoding a stable toxin and another coding for an unstable factor which prevents the lethal action of the toxin. While the toxin is always represented by a protein, the antidote is either antisense RNA, e.g. in the so-called *hok*/*sok* mechanism, or a protein (Jensen et al., 1995; Jensen and Gerdes, 1995; Engelberg-Kulka and Glaser, 1999). Meanwhile several modifications of addiction systems are used in industry for fermentative production of high-value substances (Friehs, 2004). Most of them are based on auxotrophic mutants whose complementation is combined with the expression of another gene leading to the desired product (Morsey, 1997; Brey et al., 1995; Nakayama et al., 1988). In this study we report on the construction of an effective and stabilized system for the expression of a cyanobacterial *cphA* gene in recombinant strains of *R. eutropha*, and it could be clearly shown that production of cyanophycin in *R. eutropha* is strongly affected by the expression system used. Furthermore, a different organization of *cphA* in the recombinant, synthetic operon of the addiction system in comparison to the other applied expression systems might cause a higher transcript stability in the cells, which may lead to improved expression of CphA and consequently production of cyanophycin. In comparison to unstable multi-copy plasmids or a single *cphA* gene copy strain, application of the KDPG-aldolase gene-dependent addiction system seems to be suitable for biotechnological production of cyanophycin employing recombinant strains of *R. eutropha*. Stable expression of *cphA* will be also helpful for further detailed studies aiming at improved provision of the cyanophycin synthetase with aspartic acid and especially arginine.

Acknowledgments

The project was partially carried out within the framework of the Competence Network Göttingen 'Genome Research on Bacteria' (GenoMik) financed by the German Federal Ministry of Education and Research (BMBF). The gift of a 2-keto-3-desoxy-6-phosphogluconate sample by Botho Bowien (Goerg-August-IUniversität Göttingen) is gratefully acknowledged.

References

Aboulmagd, E., Oppermann-Sanio, F.B., Steinbüchel, A., 2000. Molecular characterization of the cyanophycin synthetase of *Synechocystis* sp. strain PCC6308. Arch. Microbiol. 174, 297–306.

Aboulmagd, E., Oppermann-Sanio, F.B., Steinbüchel, A., 2001a. Purification of *Synechocystis* sp. strain PCC6308 cyanophycin synthetase and its characterization with respect to substrate and primer specificity. Appl. Environ. Microbiol. 67, 2176–2182.

Aboulmagd, E., Voss, I., Oppermann-Sanio, F.B., Steinbüchel, A., 2001b. 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.

Berg, H., Ziegler, K., Piotukh, K., Baier, K., Lockau, W., Volker-Engert, R., 2000. Biosynthesis of the cyanobacterial reserve polymer multi-L-arginyl-poly L-aspartic acid (cyanophycin). Mechanism of the cyanophycin synthetase reaction studied with synthetic primers. Eur. J. Biochem. 267, 5561–5570.

Birnboim, H.C., Doly, J., 1979. A rapid alkaline procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513–1523.

Blackkolb, F., Schlegel, H.G., 1968. Katabolische Repression und Enzymhemmung durch molekularen Wasserstoff bei *Hydrogenomonas*. Arch. Mikrobiol. 62, 129–143.

Borzi, A., 1887. Le communicazioni intracellulari delle Nostochinee. Malpighia 1, 28–74.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brey, R.N., Fulginiti, J.P., Anilionis, A., 1995. Stable *purA* vectors and uses therefore. US Patent 5,919,663.

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.

Engelberg-Kulka, H., Glaser, G., 1999. Addiction modules and programmed cell death and antideath in bacterial cultures. Annu. Rev. Microbiol. 53, 43–70.

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.

Friedrich, B., Hogrefe, C., Schlegel, H.G., 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. 147, 198–205.

Friehs, K., 2004. Plasmid copy number and plasmid stability. Adv. Biochem. Eng. Biotechnol. 86, 47–82.

Gottschalk, G., Schlegel, H.G., Eberhardt, U., 1964. Verwertung von Fruktose durch *Hydrogenomonas* H16 (I). Arch. Mikrobiol. 48, 95–108.

Hai, T., Oppermann-Sanio, F.B., Steinbüchel, A., 1999. Purification and characterization of cyanophycin and cyanophycin synthetase from the thermophilic *Synechococcus* sp. MA19. FEMS Microbiol. Lett. 181, 229–236.

Hai, T., Oppermann-Sanio, F.B., Steinbüchel, A., 2002. Cloning and characterization of a thermostable cyanophycin synthetase from the thermophilic cyanobacterium *Synechococcus* sp. MA19 and in vitro synthesis of cyanophycin and related polyamides. Appl. Environ. Microbiol. 68, 93–101.

Jendrossek, D., Kratzin, H.D., Steinbüchel, A., 1993. The *Alcaligenes eutrophus ldh* structural gene encodes a novel type of lactate dehydrogenase. FEMS Microbiol. Lett. 112, 229–236.

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

Jensen, R.B., Grohmann, E., Schwab, H., Diaz-Orejas, R., Gerdes, K., 1995. Comparison of *ccd* of F, *parDE* of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. 17, 211–220.

Joentgen, W., Groth, T., Steinbüchel, A., Hai, T., Oppermann, F. B., 1998. Polyaspartic acid homopolymers and copolymers, biotechnical production and use thereof. Patent application WO 98/39090.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop II, R.M., Peterson, K.M., 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175–176.

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

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

Mackerras, A.H., de Chazal, N.M., Smith, G.D., 1990. Transient accumulation of cyanophycin in *Anabaena cylindrica* and *Synechocystis* 6308. J. Gen. Microbiol. 136, 2057–2065.

Marmur, J., 1961. A procedure for the isolation of desoxyribonucleic acid from microorganisms. J. Mol. Biol. 1, 208–218.

Morsey, M.A., 1997. Methods for production of recombinant plasmids. Patent application WO 97/14805.

Nakayama, K., Kelly, S.M., Curtiss, R., 1988. Construction of an *asd*$^+$ expression-cloning vector—stable maintenance and high-level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology 6, 693–697.

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.

Ohta, K., Beall, D.S., Mejia, J.P., Shanmugam, K.T., Ingram, L.O., 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57, 893–900.

Oppermann-Sanio, F.B., Hai, T., Aboulmagd, E., Hezayen, F.F., Jossek, S., Steinbüchel, A., 1999. Biochemistry of microbial polyamide metabolism. In: Steinbüchel, A. (Ed.), Biochemical Principles and Mechanisms of Biosynthesis and Biodegradation of Polymers. Wiley-VCH, Weinheim, pp. 185–193.

Overhage, J., Priefert, H., Rabenhorst, J., Steinbüchel, A., 1999. Biotransformation of eugenol to vanillin by a mutant of *Pseudomonas* sp. strain HR199 constructed by disruption of the vanillin dehydrogenase (*vdh*) gene. Appl. Microbiol. Biotechnol. 52, 820–828.

Pötter, M., Müller, H., Steinbüchel, A., 2005. Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in *Ralstonia eutropha* H16. Microbiology 151, 825–833.

Richter, R., Hejazi, M., Kraft, R., Ziegler, K., Lockau, W., 1999. Cyanophycinase, a peptidase degrading the cyanobacterial reserve material multi-L arginyl-poly-L-aspartic acid (cyanophycin). Molecular cloning of the gene of *Synechocystis* sp. PCC6803, expression in *Escherichia coli*, and biochemical characterization of the purified enzyme. Eur. J. Biochem. 263, 163–169.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Moleular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schlegel, H.G., Gottschalk, G., 1965. Verwertung von Glukose durch eine Mutante von *Hydrogenomonas* H16. Biochem. Z. 341, 249–259.

Schlegel, H.G., Kaltwasser, H., Gottschalk, G., 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38, 209–222.

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

Schwartz, E., Friedrich, B., 2001. A physical map of the megaplasmid pHG1, one of the three genomic replicons in *R. eutropha* H16. FEMS Microbiol. Lett. 201, 213–219.

Schweizer, H., Hoang, T.T., 1995. An improved system for gene replacement and *xyl*E fusion analysis in *Pseudomonas aeruginosa*. Gene 158, 12–22.

Simon, R.D., 1976. The biosynthesis of multi-L-arginyl-poly(L-aspartic acid) in the filamentous cyanobacterium *Anabaena cylindrica*. Biochim. Biophys. Acta 42, 407–418.

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.

Simon, R., Priefer, U., Pühler, A., 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology 1, 784–791.

Srinivasan, S., Barnard, G.C., Gerngross, T.U., 2003. Production of recombinant proteins using multiple-copy gene integration in high cell density fermentations of *Ralstonia eutropha*. Biotechnol. Bioeng. 84, 114–120.

Steinbüchel, A., 1986. Expression of the *Escherichia coli pfkA* gene in *Alcaligenes eutrophus* and other Gram-negative bacteria. J. Bacteriol. 166, 319–327.

Steinbüchel, A., Kuhn, M., Niedrig, M., Schlegel, H.G., 1983. Fermentation enzymes in strictly aerobic bacteria: comparative studies on strains of the genus *Alcaligenes* and *Nocardia opaca* and *Xanthobacter autotrophicus*. J. Gen. Microbiol. 129, 2825–2835.

Voss, I., Steinbüchel, A., 2001. High cell density cultivation of *Rhodococcus opacus* for lipid production at a pilot-plant scale. Appl. Microbiol. Biotechnol. 55, 547–555.

Voss, I., Diniz Cardoso, S., Aboulmagd, E., Steinbüchel, A., 2004. Identification of the *Anabaena* sp. strain PCC7120 cyanophycin synthetase as suitable enzyme for production of cyanophycin in Gram-negative bacteria like *Pseudomonas putida* and *Ralstonia eutropha*. Biomacromolecules 5, 1588–1595.

Zabriskie, D.W., Arcuri, E.J., 1986. Factors influencing productivity of fermentations employing recombinant microorganisms. Enzyme Microb. Technol. 8, 706–717.

Ziegler, K., Diener, A., Herpin, C., Richter, R., Deutzmann, R., Lockau, W., 1998. Molecular characterization of cyanophycin synthetase, the enzyme catalyzing the biosynthesis of the cyanobacterial reserve material multi-L-arginyl-poly L-aspartate (cyanophycin). Eur. J. Biochem. 254, 154–159.

Ziegler, K., Deutzmann, R., Lockau, W., 2002. Cyanophycin synthetase-like enzymes of non-cyanobacterial eubacteria: characterization of the polymer produced by a recombinant synthetase of *Desulfitobacterium hafniense*. Z. Naturforsch. 57, 522–529.