APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2008, p. 3410–3418 Vol. 74, No. 11
0099-2240/08/$08.00+0 doi:10.1128/AEM.00366-08

Synthesis and Accumulation of Cyanophycin in Transgenic Strains of *Saccharomyces cerevisiae*▽

Anna Steinle,[1] Fred Bernd Oppermann-Sanio,[1] Rudolf Reichelt,[2] and Alexander Steinbüchel[1]*

Institut für Molekulare Mikrobiologie und Biotechnologie[1] *and Institut für Medizinische Physik und Biophysik,*[2] *Westfälische Wilhelms-Universität, Münster, Germany*

Received 13 February 2008/Accepted 4 April 2008

Cyanophycin [multi-L-arginyl-poly(L-aspartic acid) (CGP)] was, for the first time, produced in yeast. As yeasts are very important production organisms in biotechnology, it was determined if CGP can be produced in two different strains of *Saccharomyces cerevisiae*. The episomal vector systems pESC (with the galactose-inducible promoter *GAL1*) and pYEX-BX (with the copper ion-inducible promoter *CUP1*) were chosen to express the cyanophycin synthetase gene from the cyanobacterium *Synechocystis* sp. strain PCC 6308 ($cphA_{6308}$) in yeast. Expression experiments with transgenic yeasts revealed that the use of the *CUP1* promoter is much more efficient for CGP production than the *GAL1* promoter. As observed by electrophoresis of isolated CGP in sodium dodecyl sulfate-polyacrylamide gels, the yeast strains produced two different types of polymer: the water-soluble and the water-insoluble CGP were observed as major and minor forms of the polymer, respectively. A maximum CGP content of 6.9% (wt/wt) was detected in the cells. High-performance liquid chromatography analysis showed that the isolated polymers consisted mainly of the two amino acids aspartic acid and arginine and that, in addition, a minor amount (2 mol%) of lysine was present. Growth of transgenic yeasts in the presence of 15 mM lysine resulted in an incorporation of up to 10 mol% of lysine into CGP. Anti-CGP antibodies generated against CGP isolated from *Escherichia coli* TOP10 harboring $cphA_{6308}$ reacted with insoluble CGP but not with soluble CGP, if applied in Western or dot blots.

Cyanophycin, also referred to as multi-L-arginyl-poly(L-aspartic acid) or cyanophycin granule polypeptide (CGP), is a nonribosomally synthesized polypeptide consisting of a poly(aspartic acid) backbone with arginine residues linked to the β-carboxyl group of each aspartate by the α-amino group (45). CGP is a polydispersed polymer; the molecular size distribution of CGP varies with the producing host strains (1, 15, 18, 20, 30, 37, 52). Due to its branched structure, CGP is not degradable by a wide range of proteinases (45). Biosynthesis of CGP from aspartate and arginine requires only one enzyme, cyanophycin synthetase, which is encoded by *cphA* (51). CGP is insoluble at neutral pH and under physiological ionic strength, but it is soluble at low (>3) or high (<9) pH. Ziegler et al. were the first to observe a water-soluble form of CGP after the heterologous expression of *cphA* from *Desulfitobacterium hafniense* strain DSM 10664 in *Escherichia coli* (52). A detailed study of the solubility behavior of CGP isolated from recombinant *E. coli* in inorganic salts has been carried out by Füser and Steinbüchel (16). It was shown that the occurrence of the soluble form was not dependent on the origin of *cphA* or on the host.

Recently, several putative applications for CGP and its derivatives have become available, indicating there is a need for its efficient biotechnological production (36, 42, 43). For the production of CGP at a technical scale, cyanobacteria were shown to be unsuitable due to their low cell densities and polymer contents (3.5% [wt/wt]) and slow growth and circumstantial growth conditions in a photobioreactor (18, 19). In contrast, much higher amounts of the polymer were produced with the heterotrophic bacterium *Acinetobacter baylyi* strain ADP1 (46% [wt/wt]) (14) and with recombinant strains of *E. coli* (24% [wt/wt] [15] and 34.5% [wt/wt] [21], respectively). Also, in the industrially relevant bacteria *Pseudomonas putida*, *Ralstonia eutropha*, and *Corynebacterium glutamicum*, considerable amounts of CGP could be produced after heterologous expression of *cphA* (3, 13, 48, 49). Recently, CGP production was also achieved for the first time in eukaryotic organisms. Transgenic tobacco plants accumulated up to 1.14% (wt/wt) and transgenic potato plants up to 0.24% (wt/wt) of water-soluble and water-insoluble CGP (37).

In the last century, yeasts have evolved into biotechnologically relevant production organisms for several products in industry. First, yeast systems that were developed for heterologous gene expression were based on *Saccharomyces cerevisiae*. This organism is traditionally used for large-scale production of baker's yeast and ethanol, with a considerable increase in the production of fuel ethanol in the last 3 decades (4, 6, 50). Additionally, this platform has been successfully applied to the production of valuable heterologous proteins on an industrial scale (26, 35), such as various FDA-approved pharmaceuticals, including insulin (34) and hepatitis B surface antigen (23). Yeast-based expression systems excel because of their available constitutive or strongly inducible promoters and their growth to high cell densities on inexpensive substrates. The range of today's established yeast expression systems includes *S. cerevisiae*, *Kluyveromyces lactis*, *Pichia pastoris*, *Yarrowia lipolytica*, *Arxula adeninivorans*, and *Hansenula polymorpha* (8, 17). Polymers such as human collagen and recombinant gelatin have also been produced successfully in several yeast strains (7,

* Corresponding author. Mailing address: Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität, Münster, Corrensstraße 3, D-48149 Münster, Germany. Phone: 49 251 8339821. Fax: 49 251 8338388. E-mail: steinbu@uni-muenster.de.
▽ Published ahead of print on 11 April 2008.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	11
E. coli TOP10	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ*lacU169*	Invitrogen
E. coli BL21(DE3)	F′ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
S. cerevisiae G175	*MAT*α *ADE2 MET his3 leu2 ura3 trp1* TAG$^+$ SE$^+$	Scandinavian Biotechnology Research (41)
S. cerevisiae BY4741	*MAT*α *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	9
pSK::*cphA*$_{co}$	Ap^r; *cphA* from *Synechocystis* sp. strain PCC 6308 cloned as an HpaI fragment into pBluescript SK$^-$ (Stratagene) colinear to the *lac* mutant promoter	1
pET-23a::*cphA*$_{6308}$	Ap^r; *cphA* from *Synechocystis* sp. strain PCC 6308 cloned as an NdeI-XhoI fragment into pET-23a (Novagen) colinear to the T7 promoter	Steinle et al., unpublished data
pESC-URA	*E. coli-S. cerevisiae* shuttle vector; *URA3 GAL1 GAL10* Ap^r	Stratagene
pYEX-BX	*E. coli-S. cerevisiae* shuttle vector; *URA3 LEU2 CUP1* Ap^r	Clontech Laboratories
pESC-URA::*cphA*$_{6308}$	*cphA* from *Synechocystis* sp. strain PCC 6308 with an artificial Kozak initiation sequence cloned as a 2.6-kbp BamHI-SalI fragment into pESC-URA colinear to the *GAL1* promoter	This study
pYEX-BX::*cphA*$_{6308}$	*cphA* from *Synechocystis* sp. strain PCC 6308 with an artificial Kozak initiation sequence cloned as a 2.6-kbp BamHI-SalI fragment into pESC-URA colinear to the *CUP1* promoter	This study

[a] Ap^r, ampicillin resistance; Tc^r, tetracycline resistance.

12, 38, 47). The present study describes for the first time the expression of a cyanophycin synthetase gene and the successful production of CGP in yeast. These experiments emphasize the potential for biotechnological production of CGP in these industrially relevant organisms.

MATERIALS AND METHODS

Strains, media, and growth conditions. All bacteria, yeast strains, and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue was used for plasmid maintenance and propagation and was grown in Luria-Bertani (LB) medium containing ampicillin (100 mg/liter) overnight at 37°C and agitated at 300 rpm. Yeast strains were cultivated in a rich YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose) or in selective medium (0.67% [wt/vol] amino-acid-free Difco yeast nitrogen base containing 2% [wt/vol] galactose or glucose and supplemented as required with L-leucine [100 mg/liter], L-methionine [20 mg/liter], L-histidine [20 mg/liter], and L-tryptophan [20 mg/liter]). Solid medium contained 2% (wt/wt) agar for the growth of yeasts and *E. coli*. Yeasts were grown in 50-ml Erlenmeyer flasks for 12 to 48 h at 30°C and agitated at 300 rpm.

Transfer of DNA. Competent cells of *E. coli* were obtained using the $CaCl_2$ procedure (40), and transformation of the cells was carried out according to the method described by Hanahan (22). Yeast transformation was performed by the lithium acetate procedure (28). Transformants were selected on minimal glucose medium lacking either uracil or leucine but supplemented for the other auxotrophic requirements of the respective yeast strain.

General DNA techniques. Isolation of plasmids from *E. coli* was carried out using the alkaline lysis method described by Sambrook et al. (40). For DNA endonuclease digestion, standard ligation and agarose gel electrophoresis protocols were used (40). For the recovery of DNA fragments after electrophoresis, an Eppendorf Perfectprep gel clean-up kit was used by following the manufacturers instructions. Sequences of constructs were verified by capillary DNA sequencing (Universitätsklinikum, Münster, Germany). Isolation of total DNA from transgenic yeast was performed according to a method described by Kaiser et al. (29). For verification of the presence of *cphA*$_{6308}$, PCRs with specific oligonucleotides (5′-AAAAGGATCCACTATGAAAATCCTCAAAACACAA ACCC-3′ and 5′-TTTGTCGACCTATTCACTACTGAGATGATATTTCTCA ATCATC-3′) as primers and with total DNA from transgenic yeasts as templates were carried out.

Cloning of *cphA*. For cloning *cphA*$_{6308}$ into the *E. coli*-yeast shuttle vectors pESC-URA and pYEX-BX (Table 1), PCR was done with *Pfx* DNA polymerase (Gibco BRL) according to the manufacturer's instructions, by using the oligonucleotides *cphA*-fw-BamHI (5′-AAAGGATCCACTATGAAAATCCTCAAA ACACAAACCC-3′) as the sense and *cphA*-rw-SalI (5′-TTTGTCGACCTATT CACTACTGAGATGATATTTCTCAATCATC-3′) as the reverse primers. Thereby, an artificial Kozak site (underlined) upstream of the start codon, a BamHI restriction site in the upstream region and a SalI restriction site in the downstream region of *cphA*$_{6308}$, were introduced. Plasmid pET-23a::*cphA*$_{6308}$ (Table 1) was used as the template. Subsequently, the PCR products were cloned into the BamHI-SalI-treated *E. coli*-yeast shuttle vectors, yielding pESC-URA::*cphA*$_{6308}$ and pYEX-BX::*cphA*$_{6308}$, respectively.

Cell disruption and determination of CDM. Yeast cells were harvested by using a bench centrifuge (5 min, 3,000 rpm, 4°C), and cell pellets were washed once with saline (0.9% [wt/vol] NaCl). For determination of the cell dry matter (CDM), pellets were lyophilized, and the cell mass was determined gravimetrically. For cell disruption, the cell pellet was resuspended in 1 ml buffer (20 mM Tris-HCl [pH 7.5]) per g of 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, 13,000 rpm, 4°C).

Determination of protein concentrations. Protein concentrations were determined using the methods described by Bradford (10) and Lowry et al. (32). Soluble cell fractions were used for the determination and were obtained as described above.

Cyanophycin synthetase assay. The cyanophycin synthetase enzyme assay followed the procedure described by Aboulmagd et al. (1). Soluble cell fractions and crude cell extracts were used to determine the enzyme activity. Scintillation counting was carried out using a model LS 6500 scintillation counter (Beckman Instruments GmbH, München, Germany).

Isolation of CGP. For the isolation of CGP, yeast cells were disrupted as described above. The crude cell extract obtained after cell disruption was centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was used for the isolation of water-soluble CGP by applying a modified method described by Ziegler et al. (52), using heat treatment, proteinase K digestion, and precipitation with 3 volumes of ethanol. After samples were subjected to proteinase K digestion, they were applied to Vivaspin 20 concentrators (Vivascience AG, Hannover, Germany), with a 10-kDa membrane to remove low-molecular-weight substances. CGP was subsequently precipitated with ethanol and washed once with acetone. Water-insoluble CGP was isolated from the cell debris by resuspending the sample in 0.1 M HCl. After the suspension was centrifuged (15 min, 13,000 rpm), the supernatant was neutralized by adding NaOH. After another centrifugation step, the polymer was obtained from the pellet and washed twice with demineralized water and lyophilized to determine the dry weight. Isolation of CGP from *E. coli* BL21(DE3)(pET-23a::*cphA*$_{6308}$) was performed as described by Frey et al. (15).

Characterization of CGP. The amino acid constituents of the water-soluble and water-insoluble CGP isolated from the transgenic yeasts G175(pESC-URA::*cphA*$_{6308}$), G175(pYEX-BX::*cphA*$_{6308}$), and BY4741(pYEX-BX::*cphA*$_{6308}$) were determined by high-performance liquid chromatography (HPLC) (1). Calibration was done with samples from an amino acid reference kit (Kollektion AS-10 from Serva Feinbiochemica, Heidelberg, Germany).

Electrophoretic methods. Analysis of polymers by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 11.5% (wt/vol) acrylamide gels according to the method described by Laemmli (31). Prestained standard molecular weight 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). Proteins and CGP-like material were stained with Serva Blue R. Protein concentrations were determined as described by Hartree (24).

Immunological methods. For the preparation of anti-CGP antiserum, cells of *E. coli* TOP10(pSK::$cphA_{co}$) (Table 1) were grown in LB medium under ampicillin selection (100 μg/ml). After the cells were harvested by centrifugation for 15 min at 4°C, they were washed once with 0.9% (wt/vol) NaCl and disrupted by using a French pressure cell with 1,000 mPa (Amicon, Silver Spring, MD). Subsequently, CGP was isolated according to the method described by Simon (44). Purified CGP (100 mg/ml) was used for the generation of custom polyclonal antibodies in rabbits by Eurogentec (Seraing, Belgium). The immunoglobulin G (IgG) fraction was purified by affinity chromatography using protein A-Sepharose CL-4B (27). To purify anti-CGP-specific IgG, CGP was blotted onto a Hybond-P membrane (Amersham Biosciences). The membrane was incubated in 2.5% (wt/vol) skim milk in TBS buffer (8% [wt/vol] NaCl; 2% [vol/vol] Tris/HCl [pH 7.6]) for 1 h. After the membrane was washed three times for 10 min each in TBS, 400 μl of the IgG solution from protein A chromatography was added. After a 3-h incubation, the membrane was washed again, and anti-CGP-specific IgGs were eluted with 1 ml of elution buffer (5 mM glycine [pH 3], 0.5 M NaCl, 0.05% [vol/vol] Tween 20), neutralized with 1 M potassium phosphate buffer (pH 8.0), and stored at −20°C. For immunological detection of CGP, proteins and CGP were transferred from gels onto Hybond-P membranes as described by the manufacturer (Amersham Biosciences). Immunological analysis was performed as described in reference 25 with slight modifications, employing anti-CGP-specific IgGs (diluted 1:100 in TBS buffer). Dot blot experiments were performed as described by the manufacturer of the polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, United Kingdom). IgGs were visualized on immunoblots by using anti-rabbit IgG–alkaline phosphatase conjugates (Sigma-Aldrich), converting 5-bromo-4-chloro-3-indolyl-phosphate dipotassium nitrotetrazolium blue chloride (Sigma-Aldrich) into a dark insoluble product.

Isolation of RNA and RT-PCR. For isolation of RNA, yeast cells were grown in 5 ml of selective medium under induced conditions. Therefore, cells harboring the vector pESC-URA::$cphA_{6308}$ were grown in minimal medium with 2% (wt/vol) galactose as the inducer for the *GAL1* promoter and the carbon source, and cells harboring pYEX-BX::$cphA_{6308}$ were grown in minimal medium containing 0.1 mM $CuSO_4$ as the inducer for the *CUP1* promoter and 2% (wt/vol) glucose as the carbon source. Cells were harvested (3 min, 5,000 rpm) and broken by treatment in a bead mill (type MM 301; Retsch, Haan, Germany). RNA was isolated by using an RNeasy mini-kit (Qiagen, Hilden, Germany) as described by the manufacturer. After RNA was isolated, the remaining DNA was hydrolyzed by DNaseI (Roche Diagnostics, Mannheim, Germany) during an incubation of 45 min at 37°C. To determine if the *cphA* gene was transcribed in the transgenic yeast cells, reverse transcription-PCR (RT-PCR) was performed as described by the manufacturer (OneStep RT-PCR kit; Qiagen, Hilden, Germany), using *cphA*-specific oligonucleotides (5′-GCCATCGCTGATGTCGGTGG-3′ and 5′-CGATGGCAATACCCCCGGTAC-3′) as the primers and 0.5 ng RNA as the template. DNA controls were carried out to exclude any DNA contamination.

Transmission electron microscopy (TEM) studies. Cells were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 45 min. After the cells were washed three times with PBS for 20 min each, they were postfixed in 1% (wt/vol) 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, and 96%, and absolute ethanol) in which each step lasted about 15 min. To obtain thin sections, the samples were embedded in Spurr resin without propylene oxide (46). Sections with a thickness of 70 to 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 a lead citrate solution according to the method described by Reynolds (39) for 3 min. Imaging was performed with an H-500 model TEM (Hitachi, Tokyo, Japan) in the brightfield mode at an acceleration voltage of 80 kV at room temperature.

RESULTS

Generation of transgenic yeasts. The cyanophycin synthetase gene carried by *Synechocystis* sp. strain PCC 6308 ($cphA_{6308}$) was amplified by PCR and ligated into two different *E. coli*-yeast shuttle vectors to compare the suitabilities of two different inducible promoters for the expression of $CphA_{6308}$ and the establishment of CGP biosynthesis in *S. cerevisiae*. In vector pESC, $cphA_{6308}$ is under the control of a galactose-inducible promoter (*GAL1*), whereas in vector pYEX-BX, $cphA_{6308}$ is controlled through the *CUP1* promoter, which is induced by the addition of 0.1 mM copper ions. Whereas vector pYEX-BX::$cphA_{6308}$ mediated uracil and also leucine prototrophy, vector pESC-URA mediated only uracil prototrophy. *S. cerevisiae* strains G175 and BY4741 (Table 1) were transformed with both generated plasmids (Table 1), yielding transgenic yeast strains G175(pESC-URA::$cphA_{6308}$), BY4741 (pESC-URA::$cphA_{6308}$), G175(pYEX-BX::$cphA_{6308}$), and BY4741 (pYEX-BX::$cphA_{6308}$). In addition, the vectors without insertions were also transformed into both yeast strains. When total DNA was isolated from cells of the resulting clones, and when PCR was carried out with *cphA*-specific primers, all the obtained transformants, except for those transformed with only the vectors, showed specific PCR products, verifying the presence of *cphA* (data not shown).

Transcriptional analysis of transgenic yeast for *cphA*. For expression experiments, cells of transgenic yeasts harboring the plasmid pESC-URA::$cphA_{6308}$ were grown in minimal medium containing 2% (wt/vol) galactose as the sole carbon source and as the inducer for the *GAL1* promoter. In contrast, cells of the transgenic yeasts harboring plasmid pYEX-BX::$cphA_{6308}$ were grown in minimal medium containing 2% (wt/vol) glucose as the carbon source and 0.1 mM $CuSO_4$ as the inducer for the *CUP1* promoter. To determine whether $cphA_{6308}$ is transcribed in the transgenic yeast cells, RNA was isolated, and RT-PCR was performed. All the obtained transgenic yeasts that were transformed with *cphA*-containing plasmids showed *cphA*-specific PCR products, in contrast to that of the DNA control, indicating that *cphA* is transcribed in these cells (data not shown). Negative controls (strains G175 and BY4741 harboring only the vectors) were also analyzed; they did not show *cphA*-specific PCR products.

Analysis of CphA enzyme activity in transgenic yeasts. CphA enzyme activity was measured in soluble cell fractions obtained from *S. cerevisiae* strains G175 and BY4741 harboring pESC-URA::$cphA_{6308}$, pYEX-BX::$cphA_{6308}$, or the respective vector controls by employing a radiometric assay. The disintegrations per minute (dpm) were determined to be below 75 dpm for vector controls and the strains BY4741 and G175 harboring pESC-URA::$cphA_{6308}$, indicating the absence of significant CphA enzyme activity and that pESC-URA::$cphA_{6308}$ did not confer considerable CphA activity on the strains. In contrast, measurements in soluble cell fractions of strains BY4741 or G175 harboring pYEX-BX::$cphA_{6308}$ gave 756 or 931 dpm, respectively. Soluble cell fractions from *E. coli* strain BL21(DE3) harboring pET-23a::$cphA_{6308}$ were used as positive controls and gave 9,420 dpm. These data clearly demonstrated that pYEX-BX::$cphA_{6308}$ conferred CphA enzyme activity on both of the investigated yeast strains. Unfortunately, it was not possible to calculate real specific enzyme activities

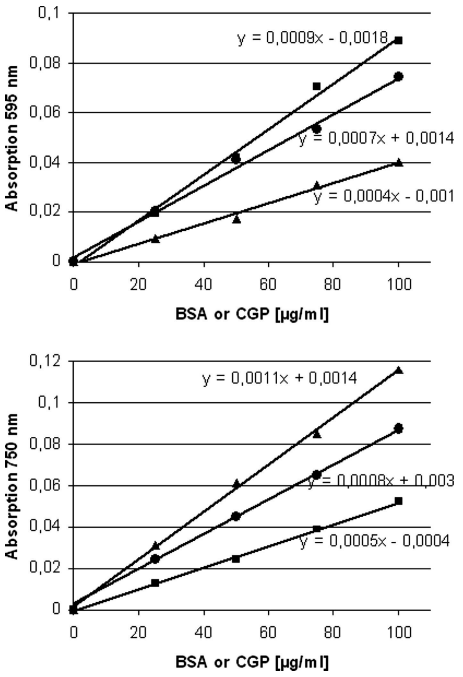

FIG. 1. Standard curves for protein analysis employing BSA (▲), soluble CGP (■), and a mixture of BSA and CGP (●). The substances were applied in concentrations of 0 to 100 μg/ml and were solubilized in water. (A) Determination according to the Bradford method (10). (B) Determination according to the method described by Lowry et al. (32). Linear equations are given in the graphs.

because protein concentrations could not be measured accurately due to the presence of CGP. The presence of CGP in protein samples affected the total protein concentration when it was determined by the Bradford method (10) or that of Lowry et al. (32). CGP gave higher protein values if determined with the Bradford reagent and quencher values if determined with the Lowry reagent, as revealed by standard curves (Fig. 1). Therefore, the real protein values could only be estimated and had to be carefully considered. After the isolation of soluble CGP, the polymer was solubilized in buffer, and the "protein" concentration of this solution was determined according to the Bradford method; the value obtained was subtracted from the protein concentration determined for the respective soluble cell fraction. The values obtained were used to calculate specific enzyme activities, which were 2.00, 0.91, and 0.76 U/mg protein for *E. coli* BL21(DE3)(pET-23a::$cphA_{6308}$), *S. cerevisiae* G175(pYEX-BX::$cphA_{6308}$), and *S. cerevisiae* BY4741(pYEX-BX::$cphA_{6308}$), respectively. Specific activities for vector controls and yeasts harboring pESC-URA::$cphA_{6308}$ were below 0.02 U/mg protein.

Analysis of cell extracts of transgenic yeasts for the presence of CGP. To determine whether the obtained transgenic yeast strains are able to synthesize CGP, we isolated the polymer by different methods and analyzed it by SDS-PAGE. For this procedure, cells were disrupted, and the crude extracts obtained were separated through centrifugation into a soluble cell fraction and the cell debris. To detect the water-soluble form of CGP, the soluble cell fractions were incubated with proteinase K. To detect the water-insoluble CGP, the cell debris fractions were incubated in 0.1 M HCl and centrifuged, and the resulting supernatant was then transferred to a new tube. Aliquots of both obtained solutions were then analyzed by SDS-PAGE (Fig. 2A). The gels clearly indicated that most transgenic yeast strains were producing considerable amounts of water-insoluble and even higher amounts of water-soluble CGP. Only analysis of the transgenic *S. cerevisiae* strain BY4741 harboring the plasmid pESC-URA::$cphA_{6308}$ did not reproducibly show any detectable amount of CGP (data not shown). Presumably, the vector pESC-URA was not suitable for the production of significant amounts of CGP in strain BY4741. SDS-PAGE revealed for the polydispersed soluble CGP a molecular mass distribution from 20 to 35 kDa, whereas for the polydispersed insoluble CGP, a slightly higher molecular mass range from about 26 to 45 kDa (Fig. 2A) was found.

Detection of CGP by anti-CGP IgGs antibodies. To have an additional tool for analysis of CGP and CGP-harboring cells, polyclonal antibodies were raised against CGP isolated and purified from a recombinant strain of *E. coli* expressing the cyanophycin synthetase from *Synechocystis* sp. strain PCC 6308, as described in Materials and Methods. Specific immunoreactions (Fig. 2A and B) occurring with Western blots from SDS-PAGE gels, in which 10 μg of CGP solubilized in HCl either from *E. coli* or *Synechocystis* sp. PCC 6308 cells was separated, indicated the functionality of these antibodies (Fig. 2B). When different amounts (1, 3, 6, 30, or 60 μg) of CGP isolated from *E. coli* BL21(DE3)(pET-23a::$cphA_{6308}$) were applied to an SDS-PAGE gel and blotted, 6 μg of CGP was the minimal detectable amount in the Western blots (data not shown).

Subsequently, the anti-CGP antibodies were applied to crude extracts prepared from transgenic yeasts cells and also to purified CGP isolated from these yeast cells (Fig. 2A). In the applied crude extracts, only CGP and no distinct protein molecules gave an immunoreaction, indicating that anti-CGP IgGs bound specifically to CGP molecules and not to other proteins (Fig. 2A). A strong immunoreaction occurred with insoluble CGP isolated from yeast as it was also observed for CGP isolated from the cells of *E. coli* or *Synechocystis* sp. Surprisingly, no immunoreaction at all occurred with the soluble CGP isolated from yeast (Fig. 2A). Due to the solubility behavior of this CGP form, blotting was also carried out by applying three membranes instead of one, to exclude the possibility that soluble CGP passed the first membrane during blotting and was therefore not detectable. However, no immunoreaction occurred on any of the three membranes (data not shown). In addition, dot blot experiments were carried out to confirm the observation that the anti-CPG antibodies used do not react with soluble CGP (Fig. 2C). Obviously, the results obtained in this experiment were the same as those in the Western blotting analysis; therefore, it was concluded that an immunoreaction occurred with insoluble CGP but not with soluble CGP (Fig. 2A and C) and that these antibodies can be used to discriminate between the two forms.

Determination of the amino acid composition. As observed by SDS-PAGE (Fig. 2A), the transgenic yeasts produced two different types of CGP which are significantly distinguishable by their solubility behavior and their reaction with the anti-CGP antibodies. Both types were isolated by different procedures, as described in Materials and Methods. HPLC analysis

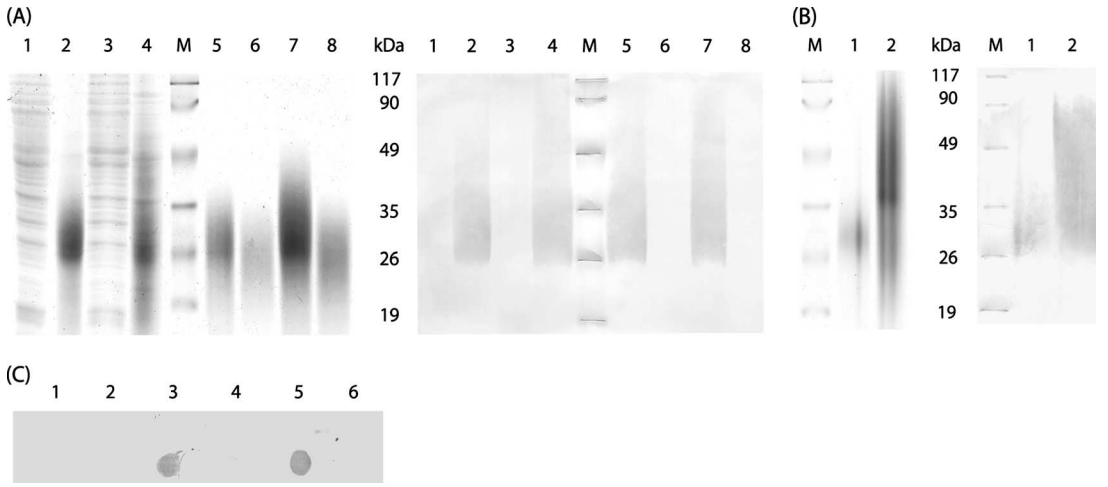

FIG. 2. Analysis and detection of CGP isolated from cells of recombinant strains of *S. cerevisiae* and *E. coli* and from cyanobacteria. (A) Yeast cell extracts were analyzed by SDS-PAGE (left) and Western blotting (right). Acidic extracts were obtained by resuspension of cell debris in 0.1 M HCl and centrifugation. Proteinase K fractions were obtained by digestion of soluble cell fractions with proteinase K. In each lane, 10 to 20 μg CGP was applied. Lanes 1 to 4 represent crude extracts from yeast: 1, BY4741 harboring pYEX-BX; 2, BY4741 harboring pYEX-BX::$cphA_{6308}$; 3, G175 harboring pYEX-BX: 4, G175 harboring pYEX-BX::$cphA_{6308}$. Lanes 5 to 8 represent soluble and insoluble CGP from yeast: 5, acidic extract from BY4741 harboring pYEX-BX::$cphA_{6308}$; 6, proteinase K fraction from BY4741 harboring pYEX-BX::$cphA_{6308}$: 7, acidic extract from G175 harboring pYEX-BX::$cphA_{6308}$; 8, proteinase K fraction from G175 harboring pYEX-BX::$cphA_{6308}$. (B) CGP isolated from *E. coli* BL21(DE3)(pET-23a::$cphA_{6308}$) and from *Synechocystis* sp. strain PCC 6308 was analyzed by SDS-PAGE (left) and by immunological detection in Western blotting (right) using anti-CGP IgGs. In each lane, 10 μg CGP was applied. M, protein marker; lane 1, CGP from recombinant *E. coli*; lane 2, CGP from *Synechocystis* sp. (C) Acidic fractions and proteinase K fractions from *S. cerevisiae* analyzed by dot blot employing anti-CGP IgGs. Lanes 1, 3 and 5, acidic fractions; 2, 4 and 6, proteinase K fractions. Lanes: 1 and 2, G175 harboring pYEX-BX; 3 and 4, G175 harboring pYEX-BX::$cphA_{6308}$; 5 and 6, BY4741 harboring pYEX-BX::$cphA_{6308}$. CGP (30 μg) was applied for *cphA*-harboring strains.

of the two CGP types revealed that both types of CGP isolated from the same cells exhibited the same amino acid compositions. CGP isolated from either the BY4741 or the G175 strain, respectively, consisted mainly of aspartic acid or arginine, which occurred at molar fractions of 52 or 46%, respectively. Lysine was detected at a maximum fraction of only 2 mol%.

Microscopy analysis of transgenic yeast. For the visualization of CGP granules in cells of *S. cerevisiae* strain G175 harboring pESC-URA::$cphA_{6308}$, phase-contrast light microscopy and electron microscopy were applied (Fig. 3B and D). *S. cerevisiae* strain G175 harboring pESC-URA was analyzed for comparison in parallel (Fig. 3A and C). Both strains were grown in minimal media containing 2% (wt/vol) galactose as the sole carbon source for 48 h at 30°C. From cells of *cphA*-carrying strains, water-soluble and water-insoluble forms of CGP were isolated in amounts similar to those described above (soluble CGP amounted to about 2% of the cell dry mass; insoluble CGP amounted to <0.1% of the cell dry mass). Since both strains showed weak light-scattering granules by light microscopy (Fig. 3C and D), electron microscopy views should have elucidated where the CGP was deposited in the cells. The micrographs revealed black areas in all investigated cells (Fig. 3A and B). Cells of strain G175 harboring pYEX-BX::$cphA_{6308}$ showed stronger light-scattering inclusions in several cells (Fig. 3E and F).

Cultivation experiments. Without varying the cultivation conditions, the total maximal CGP contents of 1.1%, 6.9%, and 5.9% (wt/wt) were measured for *S. cerevisiae* strain G175 (pESC-URA::$cphA_{6308}$), G175(pYEX-BX::$cphA_{6308}$), and BY4741 (pYEX-BX::$cphA_{6308}$), respectively (Table 2). The CDM averages were 81, 85, and 46 mg for the respective strains. CDM averages of strains harboring the vectors without inserts were 85, 106, and 81 mg for *S. cerevisiae* strain G175(pESC-URA), G175(pYEX-BX), and BY4741(pYEX-BX), respectively (Table 2). To enhance the cell densities and the amounts of polymer produced in the transgenic yeasts and to investigate the compositions of the accumulated CGP in detail, several cultivation experiments with differing conditions were performed (Table 2). Glycerol was added to the cells of *S. cerevisiae* strain G175(pESC-URA::$cphA_{6308}$) to increase the cell density. Strains B4741 and G175 harboring pYEX-BX::$cphA_{6308}$ were cultivated in the presence of different $CuSO_4$ concentrations ranging from 0.0 to 0.4 mM. Furthermore, all *cphA*-carrying cells were cultivated in the presence of the CGP constituents aspartic acid, arginine, and lysine. In addition, all transgenic yeasts were cultivated in complex medium (YPD) instead of minimal medium. All CDM values obtained and the resulting CGP contents are listed in Table 2. Obviously, cells reproducibly lost their plasmids when they were grown in complex medium, since CGP contents were drastically decreased to a maximal 0.3% insoluble CGP. Interestingly, no soluble CGP was synthesized if CGP constituents were added to the medium, but the content of insoluble CGP and the cell dry weight amount increased for all strains. The addition of glycerol in cultures of strain G175 harboring pESC-URA::$cphA_{6308}$ did not result in higher CDM values or polymer contents of the cells. This was also observed for strain G175 and strain BY4741 harboring pYEX-BX::$cphA_{6308}$ when they were grown in the presence of two- or fourfold higher concentrations of $CuSO_4$. However, HPLC analysis of CGP isolated from cells grown in the presence of 15 mM lysine revealed that this polymer consisted of up to 10 mol% of lysine.

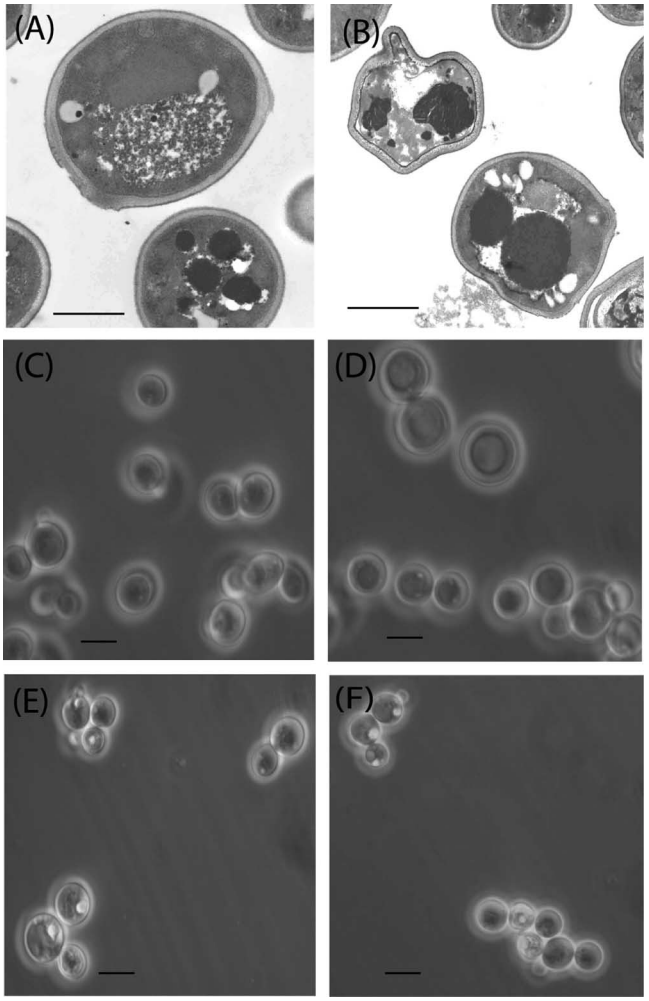

FIG. 3. Microscopy examinations of transgenic *S. cerevisiae* cells. TEM (A and B) and light microscopy (C, D, E, and F) pictures of *S. cerevisiae* G175 harboring pESC-URA (A and C) and of *S. cerevisiae* G175 harboring pESC-URA::$cphA_{6308}$ (B and D) are shown. Cells were cultivated in minimal medium containing 2% (wt/vol) galactose and harvested after 48 h. Thin sections were prepared, and electron micrographs were obtained for TEM as described in Materials and Methods. (E) A light microscopy picture of *S. cerevisiae* G175 harboring pYEX-BX::$cphA_{6308}$ grown in minimal medium containing 2% (wt/vol) glucose and 0.1 mM $CuSO_4$ and harvested after 48 h is shown. (F) A light microscopy picture of *S. cerevisiae* G175 harboring pYEX-BX::$cphA_{6308}$ is shown; cells were cultivated in minimal medium containing 2% (wt/vol) glucose and 0.4 mM $CuSO_4$ and harvested after 48 h. Bars A and B, 2 μm; bars C to F, 4 μm.

DISCUSSION

This study describes for the first time the synthesis of CGP in recombinant yeast and, besides the recently described synthesis of CGP in plants (37), the only investigation of the production of this polymer in eukaryotic organisms. It was shown that both of the investigated *S. cerevisiae* strains were able to produce the polymer in considerable amounts. However, the two applied vectors containing different promoters were not equally suitable. The vector pYEX-BX::$cphA_{6308}$ conferred synthesis of CGP to both of the *S. cerevisiae* strains, whereas strain BY4741 showed significant CGP accumulation only if it harbored the plasmid pYEX-BX::$cphA_{6308}$ but not if it harbored pESC-URA::$cphA_{6308}$. The applied vectors differ not only in their promoters but also in their auxotrophy markers. Vector pESC-URA mediates only uracil prototrophy, whereas vector pYEX-BX mediates uracil and leucine (*leu2-d*) prototrophy. However, the *leu2-d* gene present in pYEX-BX is degenerated, resulting in enhanced replication and an increase in the copy number of the whole vector, to provide enough leucine for the viability of the cells (33). The increased replication of the whole vector should result in an increased transcription of *cphA*. Cells of *S. cerevisiae* strain BY4741 and strain G175 harboring the vector pYEX-BX::$cphA_{6308}$ exhibited higher CphA enzyme activity than cells harboring vector pESC-URA::$cphA_{6308}$. This observation also correlated with the higher polymer yields in pYEX-BX::$cphA_{6308}$-harboring strains.

Immunological analyses showed that the generated anti-CGP IgGs were highly specific to CGP isolated from various organisms (Fig. 2). However, no immunoreaction occurred with water-soluble CGP synthesized by transgenic yeast (Fig. 2A). This observation was surprising as the chemical structure of this form of CGP has been reported to be identical to the water-insoluble form (16, 52); also, our analysis did not reveal any differences (data not shown). Presumably, relevant groups or regions in the CGP molecule that are recognized by the antibodies are disguised, resulting in an inability of the IgGs to bind to the soluble form. Eventually these disguised residues could also be the cause for the solubility of CGP, which is not fully elucidated yet (16). About 6 μg of CGP could be detected with Western blotting. However, this was not the real detection limit as CGP did not form a distinct band on the gel but was instead dispersed over a wide area on the gel due to its polydispersity. Nevertheless, the antibodies generated provide a suitable tool for the detection of insoluble CGP from different organisms. The molecular mass distributions of CGP was between 20 and 35 kDa for the soluble CGP and between 26 and 45 kDa for the insoluble CGP and was similar to distributions observed with recombinant bacteria and plants (3, 15, 37). In contrast, in cyanobacteria, the apparent molecular masses were much higher, ranging up to 130 kDa (18).

An interesting aspect was the amino acid composition of the isolated CGP. $CphA_{6308}$ has been described as having a broad substrate range in vitro (2) and incorporates up to 10 mol% of lysine, replacing arginine in the side chain of CGP, when it is expressed in *E. coli* (30). In contrast, CGP isolated from the natural host *Synechocystis* sp. strain PCC 6308 is composed of aspartic acid and arginine only (5). Without a variation in the medium composition, *S. cerevisiae* produced CGP with a maximum fraction of only 2 mol% of lysine. However, an increase in the amount of lysine, up to 10 mol%, was detected when 15 mM of lysine was added to the media; this composition correlated with that observed by Krehenbrink et al. (30).

Light microscopy investigations of cells harboring pESC-URA::$cphA_{6308}$ did not reveal the presence of CGP granules as they did with *A. baylyi* strain ADP1 or with recombinant *E. coli* strains (14, 15). Also, electron microscopy views did not visualize CGP granules in the yeast cells, which usually appear as black areas in the cells (14). However, such areas were widespread in the yeast strains harboring *cphA* and

TABLE 2. Cell dry weights and CGP contents determined from cultivations under different conditions[a]

Strain(plasmid)	Growth condition variable	CDM (mg)	% CGP content (mean ± SD)	
			Insoluble	Soluble
G175(pESC-URA)		85	–	–
G175(pYEX-BX)	Plus 0.1 mM $CuSO_4$	106	–	–
BY4741(pYEX-BX)	Plus 0.1 mM $CuSO_4$	81	–	–
G175(pESC-URA::*cphA*$_{6308}$)		81	0.1 ± 0.1	1 ± 1.0
	Plus glycerol 3% (wt/wt)	82	0.1 ± 0.1	1 ± 1.0
	YPD medium	249	0.1 ± 0.1	–
	Plus 10 mM Asp, 15 mM Arg	99	0.3 ± 0.1	–
	Plus 15 mM Arg	98	1.2 ± 0.2	–
	Plus 15 mM Lys	97	1.4 ± 0.2	–
G175(pYEX-BX::*cphA*$_{6308}$)	Without $CuSO_4$	88	1.3	3.1
	Plus 0.1 mM $CuSO_4$	85	2.5 ± 1.0	3.7 ± 0.2
	Plus 0.2 mM $CuSO_4$	85	2.2 ± 0.2	4.7 ± 0.2
	Plus 0.4 mM $CuSO_4$	83	1.5 ± 0.2	4.2 ± 0.3
	Plus 0.1 mM $CuSO_4$; YPD medium	288	0.1 ± 0.1	–
	Plus 0.1 mM $CuSO_4$, 10 mM Asp, and 15 mM Arg	129	2.7 ± 0.2	–
	Plus 0.1 mM $CuSO_4$ and 15 mM Arg	121	3.1 ± 0.2	–
	Plus 0.1 mM $CuSO_4$ and 15 mM Lys	83	2.2 ± 0.2	–
BY4741(pYEX-BX::*cphA*$_{6308}$)	Without $CuSO_4$	61	0.7	3.0
	Plus 0.1 mM $CuSO_4$	46	2.0 ± 1.0	3.9 ± 0.2
	Plus 0.2 mM $CuSO_4$	39	0.6 ± 0.2	3.9 ± 0.3
	Plus 0.4 mM $CuSO_4$	37	0.6 ± 0.1	4.2 ± 0.1
	Plus 0.1 mM $CuSO_4$; YPD medium	227	0.3 ± 0.1	–
	Plus 0.1 mM $CuSO_4$, 10 mM Asp, and 15 mM Arg	78	3.9 ± 0.4	–
	Plus 0.1 mM $CuSO_4$ and 15 mM Arg	75	3.3 ± 0.4	–
	Plus 0.1 mM $CuSO_4$ and 15 mM Lys	71	3.2 ± 0.4	–

[a] Cell dry weights and CGP contents are the results of cultivating *S. cerevisiae* strains BY4741 and G175 harboring vector pESC-URA or pYEX-BX or pESC-URA::*cphA*$_{6308}$ and pYEX-BX::*cphA*$_{6308}$, respectively, under different conditions. All strains were grown at 30°C for 48 h. Strains harboring pESC-URA and pESC-URA::*cphA*$_{6308}$ were grown in minimal medium containing 2% (wt/vol) galactose except where YPD medium is listed. Strains harboring pYEX-BX::*cphA*$_{6308}$ were grown in minimal medium containing 2% (wt/vol) glucose except where YPD medium is listed. Cell dry matter (CDM) amounts were determined as described in Materials and Methods. Insoluble and soluble CGP samples were isolated as described in Materials and Methods. Most cultivations were performed at least three times. Values given are means ± standard deviations (SD). –, CGP could not be isolated.

also in the negative control. As the investigated yeast strains produced mainly soluble CGP and less than 0.1% of the insoluble form, which occurs as granules, CGP could not be visualized by this method in yeast. Only *S. cerevisiae* strain G175 harboring pYEX-BX::*cphA*$_{6308}$ showed, to a larger extent, light-scattering inclusions which might be CGP granules (Fig. 3E and F).

Determination of the cell densities of transgenic yeasts harboring *cphA*$_{6308}$ yielded lower values than the negative controls without *cphA*, thereby indicating that the synthesis and accumulation of CGP resulted in slower growth. Such an inhibition due to CGP biosynthesis was recently reported in transgenic plants, too (37). However, a total CGP content of almost 7%, which was obtained without having varied the cultivation conditions, is still high in comparison to that of CGP-producing plants or some recombinant strains of *C. glutamicum* and *P. putida* (3, 37). Through slight modifications of the cultivation conditions, the CDM average was increased 1.5-fold for strains harboring pYEX-BX::*cphA*$_{6308}$, and interestingly, soluble CGP was no longer produced when the CGP constituents aspartate and arginine were added to the medium. This observation could be useful for the directed production of one form of CGP.

These experiments concerning the production of CGP in *S. cerevisiae* clearly indicated that CGP synthesis can be conferred to yeast and that these microorganisms are therefore putative candidates for the biotechnical production of CGP in the future. However, the CGP contents of the cells need to be enhanced. This could be achieved, for example, by using stronger induction systems, by engineering the metabolism of yeasts, and by varying the cultivation conditions. A chromosomal integration of *cphA* in yeast would probably be especially advantageous, thereby allowing the use of technical, low-cost media instead of specific minimal media. In addition, the use of other yeasts such as *P. pastoris* and *H. polymorpha* (17), which have become increasingly interesting for biotechnical purposes because they exhibit high levels of productivity and because they can be grown to high cell densities, could be applied in the future.

ACKNOWLEDGMENTS

We thank Ursula Malkus (Institut für Medizinische Physik und Biophysik) for expert preparation of the electron microscopic specimen. We thank Roland Klassen and Friedhelm Meinhardt (Institut für Molekulare Mikrobiologie und Biotechnologie) for providing *S. cerevisiae* strain BY4741 and vectors pESC-URA and pYEX-BX. We thank M. Gustavsson, E. Wiberg, P. Stolt, and S. Stymne (Scandinavian Biotechnology Research, Alnarp, Sweden) for providing *S. cerevisiae* strain G175. We also thank Kay M. Frey, a member of our laboratory, for providing the cyanobacterial CGP.

This project was supported by a grant (EOSLT02034) from SenterNovem (Utrecht, The Netherlands).

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