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ORIGINAL PAPER

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Evaluation of non-cyanobacterial genome sequences for occurrence of genes encoding proteins homologous to cyanophycin synthetase and cloning of an active cyanophycin synthetase from *Acinetobacter* sp. strain DSM 587

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Abstract All publicly accessible microbial genome databases were searched for the occurrence of genes encoding proteins homologous to the cyanophycin synthetase (CphA) of *Synechocystis* sp. strain PCC 6803 in order to reveal the capability of microorganisms not belonging to the cyanobacteria to synthesize cyanophycin. Among 65 genome sequences, genes homologous to *cphA* were found in *Acinetobacter* sp. strain ADP1 (encoding a protein homologous to CphA with 40% amino acid identity), *Bordetella bronchiseptica* strain RB50 (39%), *Bordetella pertussis* strain Tohama I (39%), *Bordetella parapertussis* strain 12822 (39%), *Clostridium botulinum* strain ATCC 3502 (39%), *Desulfitobacterium hafniense* strain DCB-2 (38%) and *Nitrosomonas europaea* strain ATCC 25978 (37%). The gene homologous to *cphA* from *Acinetobacter* sp. strain DSM 587 was amplified by PCR, ligated to the vector pBluescript SK$^-$ downstream of the *lac* promoter and introduced into *Escherichia coli*. The recombinant strain of *E. coli* expressed CphA activity at up to 1.2 U/mg protein and accumulated cyanophycin to up to 7.5% of the cellular dry matter, indicating that CphA of *Acinetobacter* sp. strain DSM 587 is functionally active. In *Acinetobacter* sp. strain DSM 587 itself, cyanophycin accumulated to up to 1.4% of the total protein under phosphate-limited conditions, and cyanophycin synthetase activity was detected, which indicated the function of cyanophycin as a storage compound in this strain.

Keywords Biopolymer · Cyanophycin · Cyanophycin synthetase · Cyanophycinase · CphA · CphB · *Acinetobacter* · *Bordetella bronchiseptica* · *Bordetella pertussis* · *Bordetella parapertussis* · *Clostridium botulinum* · *Desulfitobacterium hafniense* · *Nitrosomonas europaea* · *Ralstonia metallidurans* · Genome sequences

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Abbreviations *ATCC* American type culture collection · *CphA* Cyanophycin synthetase · *CphB* Cyanophycinase · *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen · *PCC* Pasteur Culture Collection of Cyanobacteria

Introduction

Cyanophycin is a branched, non-ribosomally synthesized polypeptide consisting of aspartic acid in the backbone and a roughly equimolar amount of arginine in the side chain; the arginine residues are linked to the β-carboxyl group of each aspartate by the α-amino group (Simon and Weathers 1976). Although this biopolymer from cyanobacteria was identified over 100 years ago (Borzi 1887) and occurs in most, but not all, members of this division (Allen 1984, 1988; Allen et al. 1980, Simon 1971, 1976, 1987; Lawry and Simon 1982), it has never been reported to occur in any other group of bacteria or in archaea. Therefore, textbooks of microbiology state that "cyanophycin granules are a type of storage material that is found only in cyanobacteria" (Schlegel 1993).

Cyanophycin is insoluble under physiological conditions and is accumulated in the form of membrane-less granules in the cytoplasm (Lawry and Simon 1982). Synthesis and degradation of cyanophycin in cyanobacteria are catalyzed by cyanophycin synthetase and cyanophycinase, respectively. Both enzymes are encoded by consecutive genes within a cluster, *cphA* for cyanophycin synthetase and *cphB* for cyanophycinase. Cyanobacterial cyanophycin synthetases have been purified and characterized from *Anabaena variabilis* (Ziegler et al. 1998) and the thermophilic strain *Synechococcus* sp. strain MA19 (Hai et al. 1999) as well as from recombinant *Escherichia coli* carrying *cphA* from the cyanobacteria *Synechocystis* sp. strain PCC 6803 (Ziegler et al. 1998; Oppermann-Sanio et al. 1999), *Anabaena variabilis* ATCC 29413 (Berg et al. 2000) and *Synechocystis* sp. strain PCC 6308 (Aboulmagd et al. 2000). Cyanophycin synthetases exhibit similarities both to D-alanyl-D-alanine synthetase

and mur-ligases and possess probably two ATP-binding sites. Polymerization of cyanophycin in vitro is dependent on the presence of ATP, K^+, Mg^{2+}, a cyanophycin primer and a thiol reagent such as β-mercaptoethanol in the reaction mixture (Simon 1976; Ziegler et al. 1998; Aboulmagd et al. 2001).

Cyanophycin accumulates in cyanobacteria under conditions of intense light or high carbon dioxide concentrations, phosphate or sulfur starvation, and generally during the early and mid-stationary phase (Allen et al. 1980). These results imply the role of cyanophycin as a temporary nitrogen and possibly also carbon reserve (Mackerras et al. 1990; Li et al. 2001). The high activity of cyanophycin synthetase in nitrogen-fixing heterocysts compared to vegetative cells in nitrogen-fixing *Anabaena* species further suggests that cyanophycin functions as a dynamic buffer for newly fixed nitrogen (Carr 1988; Li et al. 2001). Since cyanophycin can be considered as being an almost ideal nitrogen reserve due to its high N/C ratio, it seems unlikely that its occurrence is exclusively limited to the cyanobacteria, as stated in textbooks.

Material and methods

Bacterial strains, plasmids, DNA fragments and culture conditions

The bacterial strains, plasmids and DNA fragments used in this study are listed in Table 1. *Acinetobacter* sp. strain DSM 587 was routinely cultivated at 30 °C in complex Standard-1 medium (Merck, Darmstadt). For phosphate-depletion experiments, *Acinetobacter* sp. DSM 587 was grown in Tris-buffered medium containing 107 mM sodium glutamate, 30 mM ammonium sulfate, 50 mM Tris/HCl, (pH 7.3), 20 mM potassium chloride, 0.8 mM magnesium sulfate and 6 mg each of $FeCl_3 \cdot 6\ H_2O$, $MnCl_2 \cdot 4\ H_2O$, $ZnCl_2$, $CuSO_4 \cdot 5\ H_2O$, $CaCl_2 \cdot 2\ H_2O$ and NaCl per liter. Leucine, isoleucine and valine were added to concentrations of 0.02% (w/v). Depending on the experiment, the phosphate concentration was varied between 64 μM and 1.3 mM Na_2HPO_4. *Escherichia coli* was grown at 30 °C in Luria Bertani medium (Sambrook et al. 1986). Growth of all bacterial strains was followed by measuring the turbidity at 600 nm.

Purification and analysis of cyanophycin-like material

Cyanophycin-like material was isolated from cells of *Acinetobacter* sp. DSM 587 and recombinant *E. coli* as described by Simon (1976). The amino acid constituents of the isolated material were determined by HPLC (Aboulmagd et al. 2000).

Preparation of cell extract and assay of cyanophycin synthetase activity

Cells were harvested by centrifugation (20 min, 2,800×*g*, 4 °C), washed once with buffer (50 mM Tris HCl, pH 8.2; 20 mM KCl; 5 mM β-mercaptoethanol; 1 mM EDTA) and resuspended in 2 ml buffer per g fresh cell mass. The cells were disrupted by sonication for 1 min/ml of cell suspension using a Sonoplus GM200 sonifier (Bandelin Electronic, Berlin, Germany). The supernatant obtained after centrifugation for 15 min at 14,000×*g*, was desalted using NAP5 columns (Pharmacia, Freiburg, Germany) and used for measuring cyanophycin synthetase activity employing a radiometric assay as described by Aboulmagd et al. (2000).

Electrophoresis and determination of protein concentrations

Proteins were separated by SDS-PAGE in 11.5% acrylamide (w/v) gels as described by Laemmli (1970). Proteins and cyanophycin-like material were stained with Serva Blue R. Soluble protein concentrations were determined as described by Bradford (1976). Total protein was determined using the method of Schmidt et al. (1963).

Isolation, manipulation and transfer of DNA

Total genomic DNA was isolated from *Acinetobacter* sp. strain DSM587 by the procedure of Rao et al. (1990). Plasmid DNA was isolated from *E. coli* by alkaline lysis (Birnboim and Doly 1979). Restriction enzymes and other DNA-manipulating enzymes were used as described by the manufacturer. For transformation, competent cells of *E. coli* top10 and *E. coli* DH1 were prepared using the method of Hanahan (1983).

Analysis of nucleotide and amino acid sequences

The sequence data of databases were searched for fragments of high similarity to the translational product of *cphA* from *Synechocystis* sp. strain PCC 6803 (slr2002 from CyanoBase, Kaneko et al. 1996). All available sequence data of the DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/JGI_microbial/html/index.html>, the Institute for Genomic Research Web site at http://www.tigr.org and the Sanger centre at http://www.sanger.ac.uk/Projects/Microbes/ were searched using the BLAST program on the BLAST server of the respective Web site. The unfinished genome sequence of *Acinetobacter* sp. strain ADP1 was searched for fragments of high similarity to *cphA* of *Synechocystis* sp. strain PCC 6803 (slr2002 from CyanoBase) using the LASSAP

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
Acinetobacter sp. strain DSM 587	Auxotrophic for leucine, valine, isoleucine, derivative of *Acinetobacter* sp. strain ADP1	DSMZ
E. coli DH1	F^-, *supE44*, *hsdR17*, *recA1*, *gyrA96*, *relA1*, *endA1*, *thi-1*, λ	Hanahan (1983)
E. coli top10	F^-, *araD139*, Δ(*ara*, *leu*)7697, Δ*lacX74*, *galU*, *galk*, *rpsL*, *deoR*, Φ80*lac*ZΔM15, *endA1*, *nupG*, *recA1*, *mdrA*, Δ(*mrr hsdRMS mcrBC*)	Invitrogen (San Diego, USA)
pBluescript SK^-	Ap^r, *lacPOZ′*	Stratagene (San Diego, USA)
pSK^-::*cphA* (DSM 587)	Ap^r, *cphA* of *Acinetobacter* sp. strain DSM 587 colinear to *lacPO*	This study
pSK^-::*cphA* (PCC 6308)	Ap^r, *cphA* of *Synechocystis* sp. strain PCC 6308 colinear to *lacPO*	Aboulmagd et al. (2000)

[a]Deutsche Sammlung von Mikroorganismen und Zellkulturen

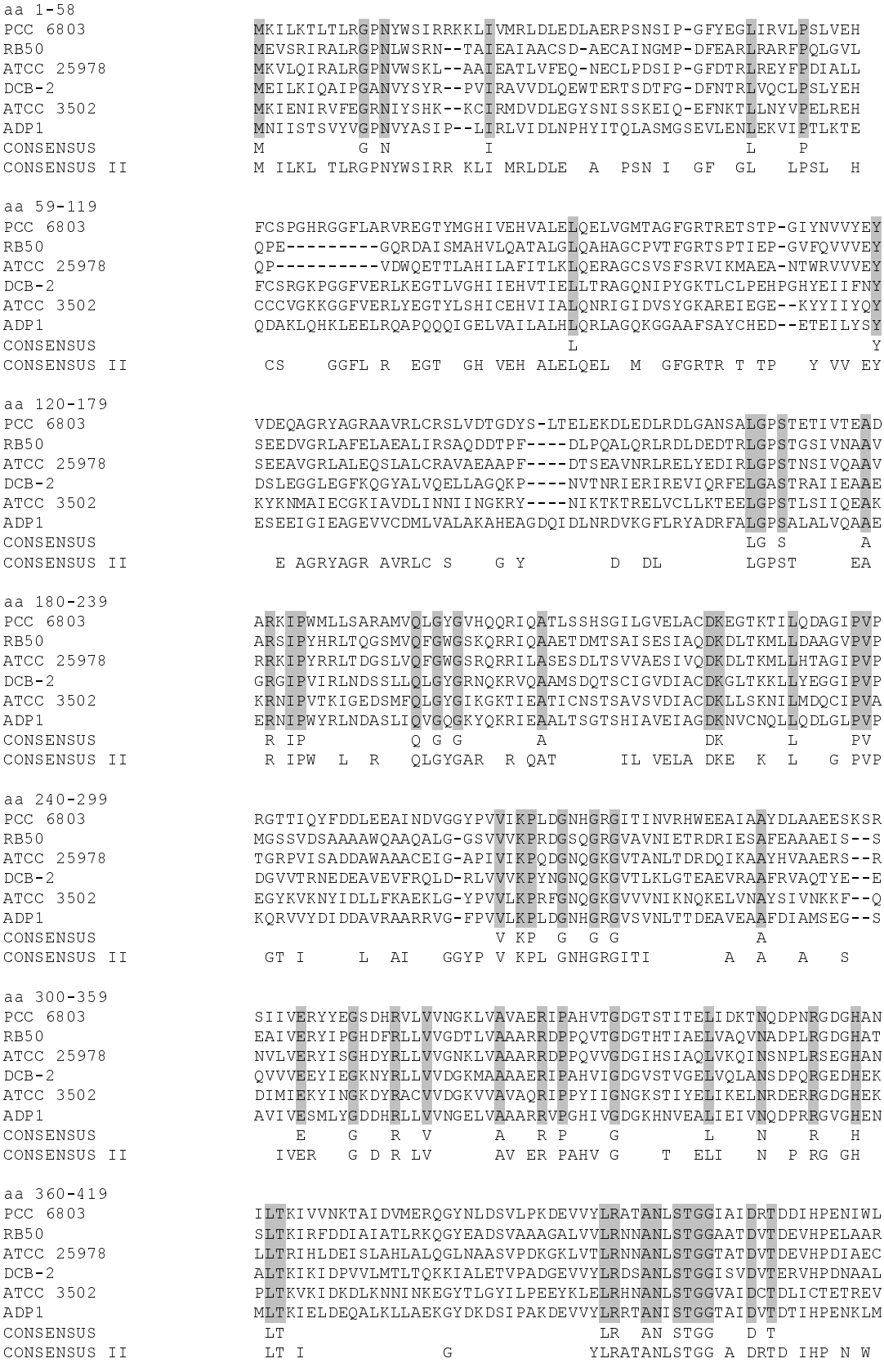
aa 1-58
PCC 6803 MKILKTLTLRGPNYWSIRRKKLIVMRLDLEDLAERPSNSIP-GFYEGLIRVLPSLVEH
RB50 MEVSRIRALRGPNLWSRN--TAIEAIAACSD-AECAINGMP-DFEARLRARFPQLGVL
ATCC 25978 MKVLQIRALRGPNVWSKL--AAIEATLVFEQ-NECLPDSIP-GFDTRLREYFPDIALL
DCB-2 MEILKIQAIPGANVYSYR--PVIRAVVDLQEWTERTSDTFG-DFNTRLVQCLPSLYEH
ATCC 3502 MKIENIRVFEGRNIYSHK--KCIRMDVDLEGYSNISSKEIQ-EFNKTLLNYVPELREH
ADP1 MNIISTSVYVGPNVYASIP--LIRLVIDLNPHYITQLASMGSEVLENLEKVIPTLKTE
CONSENSUS M G N I L L
CONSENSUS II M ILKL TLRGPNYWSIRR KLI MRLDLE A PSN I GF GL LPSL H
aa 59-119
PCC 6803 FCSPGHRGGFLARVREGTYMGHIVEHVALELQELVGMTAGFGRTRETSTP-GIYNVVYEY
RB50 QPE---------GQRDAISMAHVLQATALGLQAHAGCPVTFGRTSPTIEP-GVFQVVVEY
ATCC 25978 QP----------VDWQETTLAHILAFITLKLQERAGCSVSFSRVIKMAEA-NTWRVVVEY
DCB-2 FCSRGKPGGFVERLKEGTLVGHIIEHVTIELLTRAGQNIPYGKTLCLPEHPGHYEIIFNY
ATCC 3502 CCCVGKKGGFVERLYEGTYLSHICEHVIIALQNRIGIDVSYGKAREIEGE--KYYIIYQY
ADP1 QDAKLQHKLEELRQAPQQQIGELVAILALHLQRLAGQKGGAAFSAYCHED--ETEILYSY
CONSENSUS L Y
CONSENSUS II CS GGFL R EGT GH VEH ALELQEL M GFGRTR T TP Y VV EY
aa 120-179
PCC 6803 VDEQAGRYAGRAAVRLCRSLVDTGDYS-LTELEKDLEDLRDLGANSALGPSTETIVTEAD
RB50 SEEDVGRLAFELAEALIRSAQDDTPF----DLPQALQRLRDLDEDTRLGPSTGSIVNAAV
ATCC 25978 SEEAVGRLALEQSLALCRAVAEAAPF----DTSEAVNRLRELYEDIRLGPSTNSIVQAAV
DCB-2 DSLEGGLEGFKQGYALVQELLAGQKP----NVTNRIERIREVIQRFELGASTRAIIEAAE
ATCC 3502 KYKNMAIECGKIAVDLINNIINGKRY----NIKTKTRELVCLLKTEELGPSTLSIIQEAK
ADP1 ESEEIGIEAGEVVCDMLVALAKAHEAGDQIDLNRDVKGFLRYADRFALGPSALALVQAAE
CONSENSUS LG S A
CONSENSUS II E AGRYAGR AVRLC S G Y D DL LGPST EA
aa 180-239
PCC 6803 ARKIPWMLLSARAMVQLGYGVHQQRIQATLSSHSGILGVELACDKEGTKTILQDAGIPVP
RB50 ARSIPYHRLTQGSMVQFGWGSKQRRIQAAETDMTSAISESIAQDKDLTKMLLDAAGVPVP
ATCC 25978 RRKIPYRRLTDGSLVQFGWGSRQRRILASESDLTSVVAESIVQDKDLTKMLLHTAGIPVP
DCB-2 GRGIPVIRLNDSSLLQLGYGRNQKRVQAAMSDQTSCIGVDIACDKGLTKKLLYEGGIPVP
ATCC 3502 KRNIPVTKIGEDSMFQLGYGIKGKTIEATICNSTSAVSVDIACDKLLSKNILMDQCIPVA
ADP1 ERNIPWYRLNDASLIQVGQGKYQKRIEAALTSGTSHIAVEIAGDKNVCNQLLQDLGLPVP
CONSENSUS R IP Q G G A DK L PV
CONSENSUS II R IPW L R QLGYGAR R QAT IL VELA DKE K L G PVP
aa 240-299
PCC 6803 RGTTIQYFDDLEEAINDVGGYPVVIKPLDGNHGRGITINVRHWEEAIAAYDLAAEESKSR
RB50 MGSSVDSAAAAWQAAQALG-GSVVVKPRDGSQGRGVAVNIETRDRIESAFEAAAEIS--S
ATCC 25978 TGRPVISADDAWAAACEIG-APIVIKPQDGNQGKGVTANLTDRDQIKAAYHVAAERS--R
DCB-2 DGVVTRNEDEAVEVFRQLD-RLVVVKPYNGNQGKGVTLKLGTEAEVRAAFRVAQTYE--E
ATCC 3502 EGYKVKNYIDLLFKAEKLG-YPVVLKPRFGNQGKGVVVNIKNQKELVNAYSIVNKKF--Q
ADP1 KQRVVYDIDDAVRAARRVG-FPVVLKPLDGNHGRGVSVNLTTDEAVEAAFDIAMSEG--S
CONSENSUS V KP G G A
CONSENSUS II GT I L AI GGYP V KPL GNHGRGITI A A A S
aa 300-359
PCC 6803 SIIVERYYEGSDHRVLVVNGKLVAVAERIPAHVTGDGTSTITELIDKTNQDPNRGDGHAN
RB50 EAIVERYIPGHDFRLLVVGDTLVAAARRDPPQVTGDGTHTIAELVAQVNADPLRGDGHAT
ATCC 25978 NVLVERYISGHDYRLLVVGNKLVAAARRDPPQVVGDGIHSIAQLVKQINSNPLRSEGHAN
DCB-2 QVVVEEYIEGKNYRLLVVDGKMAAAAERIPAHVIGDGVSTVGELVQLANSDPQRGEDHEK
ATCC 3502 DIMIEKYINGKDYRACVVDGKVVAVAQRIPPYIIGNGKSTIYELIKELNRDERRGDGHEK
ADP1 AVIVESMLYGDDHRLLVVNGELVAAARRVPGHIVGDGKHNVEALIEIVNQDPRRGVGHEN
CONSENSUS E G R V A R P G L N R H
CONSENSUS II IVER G D R LV AV ER PAHV G T ELI N P RG GH
aa 360-419
PCC 6803 ILTKIVVNKTAIDVMERQGYNLDSVLPKDEVVYLRATANLSTGGIAIDRTDDIHPENIWL
RB50 SLTKIRFDDIAIATLRKQGYEADSVAAAGALVVLRNNANLSTGGAATDVTDEVHPELAAR
ATCC 25978 LLTRIHLDEISLAHLALQGLNAASVPDKGKLVTLRNNANLSTGGTATDVTDEVHPDIAEC
DCB-2 ALTKIKIDPVVLMTLTQKKIALETVPADGEVVYLRDSANLSTGGISVDVTERVHPDNAAL
ATCC 3502 PLTKVKIDKDLKNNINKEGYTLGYILPEEYKLELRHNANLSTGGVAIDCTDLICTETREV
ADP1 MLTKIELDEQALKLLAEKGYDKDSIPAKDEVVYLRRTANISTGGTAIDVTDTIHPENKLM
CONSENSUS LT LR AN STGG D T
CONSENSUS II LT I G YLRATANLSTGG A DRTD IHP N W

Fig. 1

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aa 420-479
PCC 6803              MERVAKVIGLDIAGIDVVTSDISKPLRETNGVIVEVNAAPGFRMHVAPSQGLPRNVAAPV
RB50                  AVTAARMIGLDICGVDVVAETVHLPLEDQHGGVVEVNAAPGLRMHLNPSFGKGRAVGEAI
ATCC 25978            AVMAARMTGIDICGIDVICSSLSRPLGEQGGAVIEVNAAPGLRMHLQPSYGKPRAVGEAI
DCB-2                 AEYAARIVGLDIAGVDMVLEDIERPHQEQRGAIIEVNAAPGLRMHQYPTVGRPLDVGKII
ATCC 3502             CERAAKAIGLNICGIDICCSDISKPLKENEG-IMEVNAAPGIRMHQYPYKGKSRNVAKAI
ADP1                  AERAIRAVGLDIGAVDFLTTDITKSYRDIGGGICEVNAGPGLRMHISPSEGPSRDVGGKI
CONSENSUS                    G  I   D             G   EVNA PG RMH  P  G    V
CONSENSUS II                 GLDI G D V  DI   PL    GV VEVNAAPGFRMH  PSQG  RNVA  V

aa 480-539
PCC 6803              LDMLFPSGTPSRIPILAVTGTNGKTTTRLLAHIYRQTGKTVGYTSTDAIYINEYCVEKG
RB50                  ISHMFAERDDGRIPVVAVAGTNGKTTTVRLTAHILDCAGHRVGMTNSDGVYVGKQRIDTG
ATCC 25978            IDHLFAPGENARIPVIAVTGTNGKTTTVRLIANMLENNRLRVGIACTDGVFVNGQCVDTG
DCB-2                 VDHVMP-KGNGRIPVISVTGTNGKTTTTRMIGKMLTDRELAVGMTTTDGIYVGGKLLLKG
ATCC 3502             VDMMFK-EYDGNIPIISITGTNGKTTTTRLIAHILSFSGKKVGMTTTGGIYINNKCINKG
ADP1                  MDMLFPQGSQSRVPIAAITGTNGKTTCSRMLAHILKMAGHVVGQTSTDAVYIDGNVTVKG
CONSENSUS                          P     GTNGKTT  R            VG               G
CONSENSUS II            MLFP       PI    GTNGKTTTTRL AHI  QTGQ VGYTTTD  Y      VEG

aa 540-599
PCC 6803              DNTGPQSAAVILRDPTVEVAVLETARGGILRAGLAFDTCDVGVVLNVA-ADHLGLGDIDT
RB50                  DCSGPRSARRLLLHPDVDAAVLETARGGVLREGLAFDRCNVAIVTNIGMGDHFGLGYIST
ATCC 25978            DCSGPQSARNILFHPEVDAAVLETARGGILREGLGFDYCDVAVVTNIGRGDHLGLANINT
DCB-2                 DTTGPESAQIVLRHPDVQVAVLETARGGILRAGLAYDYADVAVVTNVA-NDHLGQYGMES
ATCC 3502             DTTGYYSAKTVLTNKEAEVAVLELARGGLIRDGLPYDLADVGIITNVT-EDHLGLGGINT
ADP1                  DMTGPVSAKMVLRDPSVDIAVLETARGGIVRSGLGYQFCDVGAVLNVS-SDHLGLGGVDT
CONSENSUS             D  G  SA   L       AVLE ARGG  R GL      V    N    DH G
CONSENSUS II          D TGP SA  IL DPTVE AVLE ARGGILR GL F    VG VLNV  DHLG  GDI T

aa 600-659
PCC 6803              IEQMAKVKSVIAEVVDPS-GYAVLNADDPLVAAMADKVKAK-VAYFSMNPDNPVIQNHIR
RB50                  VEDLAVVKRVIVQYVQPD-GMAVLNAADPMVAEMASACPGS-ITYFAEDRNHPVMATHRA
ATCC 25978            AEELAAVKRTIVENVNPKTGVAVLNADDPLVLGMASHCPGN-VTFFSRNHRHPVILEQRV
DCB-2                 LEDIAHVKSLIAEVVRPH-SYVVLNADDPLVASFARKTKGK-VIFFSTEKDNLTIRKHLA
ATCC 3502             LEDMAYVKALVGEAIKKD-GYVVINADDEASISIINRMKSK-IILFTKNKNNPIISQYLD
ADP1                  LDGLAEVKRVIAEVTKDT---VVLNADNAYTLKMAGHSPAKHIMYVTRDAENKLVREHIR
CONSENSUS                 A  K               NA
CONSENSUS II            Q A  K V  E      GYA LNADD  VA M    K     YF M         H

aa 660-719
PCC 6803              RNGIAAVYES----GYVSILEGSWTLRVEEATLIPMTMGGMAPFMIANALAACLAAFVNG
RB50                  QGHRVVYRDG----DSLVAAQGG-AEVAFALADIPLTRGGAIAFQVENTMAALAAAWALG
ATCC 25978            QGKRVIYMED----HHIIVAEAG-TERRISLSQIRLTKNGMISFQIDNAMASIGAGLLAIE
DCB-2                 VGGIAVFVRR----GNILLCQGDQSHKICGVKDLPVTWNGKALHNLQNALAAIAVGWSLG
ATCC 3502             NKNLVLYLDE----DTIYLKKLNKNEEIINVNKIPITLGGKLIYNVENAMAAIAALIALG
ADP1                  LGKRAVVLEKGLNGDQIVIYENGTQIPLIWTHLIPATLEGKAIHNVENAMFAAGMAYALG
CONSENSUS                                                 T         N
CONSENSUS II              AAVYE     G  SI  G  T R E A   P T    A FMIANALAA LAAF

aa 719-779
PCC 6803              LDVEVIRQGVRTFTTSAEQTPGRMNLFNLGRYHALVDYAHNPAGYRAVGDFVKNWH--GQ
RB50                  IDWDTIRHALAIFVNDAQTAPGRFNVFDFRGATLIADYGHNPDAIQALVRAIDTMPA-RR
ATCC 25978            LDWTTICAGLADFVSDAQTVPGRFNLFNYREATLIADYGHNPDAMEALVCAIDHIPA-KK
DCB-2                 LKAEGIRTSLSEFTSDPECNRGRLNPYTIGGVQVFIDYGHNAAGIKAIAQTLRKFKA-PA
ATCC 3502             IDVNTIRQGLESFSNE-EQNPGRFNMYNVHGTNVILDYGHNIEGYKVVLESIKKIKH-KR
ADP1                  KNLDQIRIGLRTFDNTFFQSPGRMNVFDKHGFRVILDYGHNEAAVGAMTELVDRLNPRGR
CONSENSUS                  I      F        GR N           DY HN
CONSENSUS II             E IR     F     QTPGRMNLF       L DYAHN   Y A G FV  W   G

aa 780-839
PCC 6803              RFGVVGGPGDRRDSDLIELGQIAAQVFDRIIVKEDDDKRGRSGGETADLIVKGILQENPG
RB50                  RSVVISGAGDRRDEDLRQQTEILGGAFDDVILYQDQCQRGRADGEVVALLRQGLQQAPRT
ATCC 25978            RTVVISAAGDRRNEDIRLQTRILGDVFDEVVLFQDKCQRGRADGEVLGLLREGLENAKRV
DCB-2                 VVGCVTVPGDRPDETIREVARVAARGFHRLIIREDGDLRGRRPGEIAGMIMEEAIASGMD
ATCC 3502             IIGVVGVPGDRTNSSTLKVGNICGENFDYVYIKEDRDKRGRKHGEIADLLKKGILETGFK
ADP1                  RLLGVTCPGDRRDEDVVAIAAKVAGHFDEYYCHRDDDLRGRAPDETPKIMRDALIQLGVP
CONSENSUS                     GDR              F       D   RGR
CONSENSUS II          R GV GGPGDRRD D   LG      FD    KEDDD RGR  G     I  G
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Fig. 1

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aa 840-899
PCC 6803              -AAYEVILDETVALNKALDQVEEKGLVVVFPESVSKAIELIKARKPIG------------
RB50                  -RHIDEIQGEFVAIDAALERLAPGDLCLILVDQVEEALAHIASRVTGQP------------
ATCC 25978            -RKVSEIRGEFKAIDTALTNLEAGELCLILIDQVEQALGYIHSRIAVA------------
DCB-2                 PRRISVVLPEREAFCHGLDTCKPGEIFVMFYEHLEPIEEEIALRLESGPLAKEEEGFLEV
ATCC 3502             NSKLNIILDEEEALKKAIEFSNPGDLVIMFFEEFEPAENIVKDKIKKGKITKRETALA--
ADP1                  ESRIHIVEQEEDSLAAVLTEAQVDDLVLFFCENITRSWKQIVHFTPEFNIENDHETLELK
CONSENSUS                       E
CONSENSUS II             Y  I DE  A    L       LVV  P  V   I  I

aa 900-926
PCC 6803              --------------------------
RB50                  --------------------------
ATCC 25978            --------------------------
DCB-2                 ANLGAI---------------------
ATCC 3502             --------------------------
ADP1                  IAEQGFDIPEGYHAVSNDRGVMILPRG
CONSENSUS
CONSENSUS II
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Fig. 1 Sequence comparison of putative CphAs from non-cyanobacteria to CphA from *Synechocystis* sp. strain PCC 6803. Amino acid residues are specified by standard one-letter abbreviations. Gaps (–) were introduced into the sequence to improve the alignment. Amino acid residues that are identical in all six sequences are *shaded* and compiled in the line labeled *CONSENSUS*. *CONSENSUS II* shows amino acids conserved in all published cyanobacterial CphA sequences. *aa* Amino acid residue position number. The aligned CphAs are from *Synechocystis* sp. strain PCC 6803, *Bordetella bronchiseptica* strain RB50, *Nitrosomonas europaea* strain ATCC 25978, *Desulfitobacterium hafniense* strain DCB-2, *Clostridium botulinum* strain ATCC 3802, and *Acinetobacter* sp. strain ADP1

implementation of the program BLAST 1.4 at http://www.genoscope.cns.fr. The presented search results represent the data available at the mentioned databases as of August 23, 2001.

Contigs containing fragments of highest similarity were analyzed using the evaluation version of DNATools 5.1. Sequences were aligned using the program ClustalX 1.8, and phylogenetic trees were constructed using the program TREE 1.6.5.

Cloning of *cphA* from *Acinetobacter* sp. DSM 587

For amplification of *cphA* by PCR, the primers P1 (5′-CAGGGACAGTGGCCGATGAATTACT-3′) and P2 (5′-CATGGTGCTTAGTCGTTCCTGTGTG-3′) were used. Primer P1 was homologous to the region approximately 500 bp upstream of the respective gene, while P2 was homologous to the region downstream of the stop codon. Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). PCR was carried out using Pfx DNA polymerase (Gibco BRL) according to the instructions of the manufacturer. The PCR products were inserted into *Eco*RV-digested pBluescript SK$^-$ (Stratagene, San Diego, USA) using T4 DNA ligase (Gibco BRL) and transferred into *E. coli* top10.

Results

Identification of putative ORFs encoding proteins exhibiting similarity to CphA from *Synechocystis* sp. strain PCC 6803

In total, we analyzed 65 publicly available genome sequences of bacteria and archaea for the presence of ORFs whose putative translational products exhibited similarity to the cyanophycin synthetase (CphA) of *Synechocystis* sp. strain PCC 6803. High scoring ORFs were identified in *Acinetobacter* sp. strain ADP1, *Bordetella bronchiseptica* strain RB50, *Bordetella pertussis* strain Tohama I, *Bordetella parapertussis* strain 12822, *Clostridium botulinum* strain ATCC 3502, *Desulfitobacterium hafniense* strain DCB-2, and *Nitrosomonas europaea* strain ATCC 25978. An alignment of the amino acid sequences of these putative proteins with CphA from *Synechocystis* sp. strain PCC 6803 is given in Fig. 1. The identities and similarities of the putative proteins detected in the genomes of these bacteria to CphA of *Synechocystis* sp. strain PCC 6803 ranged from 35 to 40% and from 50 to 57%, respectively. For comparison, the identities and similarities of the corresponding proteins from other cyanobacteria to CphA of *Synechocystis* sp. strain PCC 6803 ranged from 63 to 74% and from 76 to 84%, respectively (Table 2). In total, 138 amino acid positions were highly conserved in any of the published cyanobacterial CphA and any non-cyanobacterial CphA revealed in this study. Figure 2 shows a dendrogram of all real and putative cyanophycin synthetases identified so far, demonstrating the coherent group of cyanobacterial CphAs. No similarities to CphA of *Synechocystis* sp. strain PCC 6803 exhibiting an expected value better than 1 e^{-100} or exhibiting similarities both to

Table 2 Amino acid sequence identity and similarity of known and putative CphAs compared to CphA from *Synechocystis* sp. strain PCC 6803

	Identity (%)	Similarity (%)	Molecular mass (kDa)
Synechocystis sp. strain PCC 6803	100	100	94.6
Cyanothece sp. strain ATCC 51142	74	84	95.2
Synechocystis sp. strain PCC 6308	71	82	94.8
Anabaena variabilis	67	78	98.2
Synechococcus elongatus	63	76	98.0
Acinetobacter sp. strain ADP1	40	57	99.6
Clostridium botulinum	39	57	97.6
Bordetella bronchiseptica CphA	39	55	91.0
Desulfitobacterium hafniense	38	56	96.4
Nitrosomonas europaea CphA	37	53	92.8
Nitrosomonas europaea CphA	35	50	78.1
Bordetella bronchiseptica CphA	35	50	94.9

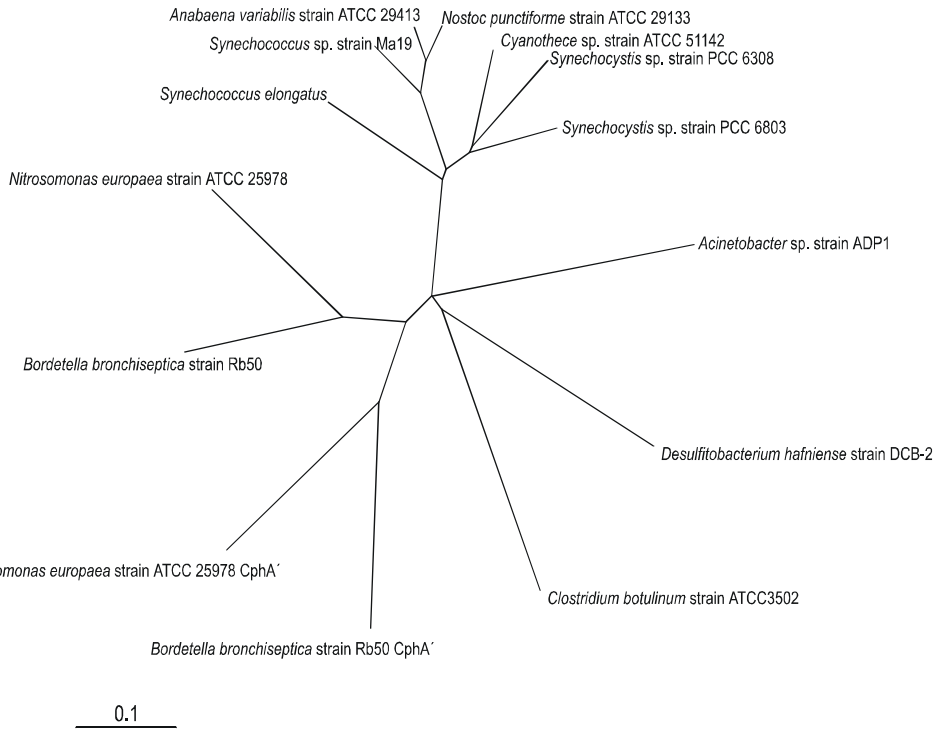

Fig. 2 Dendrogram of known and putative cyanophycin synthetases. *Bar* 0.1 per amino acid substitution

D-alanyl-D-alanine synthetase and mur-ligases were found in any of the other genomes available at the examined databases up to August 23, 2001. Similarities to CphA encoded by a DNA fragment (loe-6, GenBank AF336867) from *Sinorhizobium meliloti* strain 1021 (Trzebiatowski et al. 2001) were also examined. This 581-bp fragment is identical to an ORF encoding a hypothetical 595-amino-acid protein from *S. meliloti* strain 1021 (NCBI protein CAC48864). This protein exhibited similarities to D-alanyl-D-alanine synthetase and to only the N-terminal part of CphA, but not to mur-ligases or to the C-terminal part of CphA. However, it exhibited 51% identity and 65% similarity to a probable acetyltransferase from *Pseudomonas aeruginosa* strain PAO1 (NCBI protein C83214).

Analysis of the immediate vicinity of the genes coding for protein similar to ChpA revealed three different genetic organizations of putative *cphA* genes (Fig. 3). The gram-positive anaerobic bacteria *D. hafniense* strain DCB-2 and *C. botulinum* strain ATCC 3502 displayed the same arrangement as cyanobacteria, and *cphA* was preceded by an ORF encoding for cyanophycinase, *cphB*. In the β-Proteobacteria *N. europaea* strain ATCC 25978 and *Bordetella* sp., the putative *cphA* was preceded by a second putative ORF encoding for a protein with similarity to CphA. As these proteins lack certain sequence motifs that are conserved in all other putative and cyanobacterial CphAs, this ORF is subsequently referred to as CphA′. As the sequences of CphA′ as well as of CphA varied only to a maximum of five amino acid residues from species to species in the genus *Bordetella*, only the sequences of *B. bronchiseptica* are shown as representatives for this genus in the alignments. Notably, the sequence of CphA′ from *B. parapertussis* was identical to that from *B. bronchiseptica* except for a frameshift at amino acid residue 386. This frameshift may be existent or merely a sequencing artifact. The genetic organization *cphA′A* was also found in *Ralstonia metallidurans* CH34. However, in this strain, both ORFs were disrupted by frameshifts, and parts of the genes were significantly different from known CphAs. The third type of organization was found only in *Acinetobacter* sp. strain ADP1. In this strain, *cphA* is followed by an ORF encoding for an 80-kDa protein that resembles a fusion protein of two cyanobacterial cyanophycinases (CphB) linked to each other by a short stretch of amino acids. In the N-terminal region of this protein, which is referred to as CphI, the hypothetical catalytic triad Ser-Glu-His proposed by Richter et al. (1999) is conserved (Fig. 4).

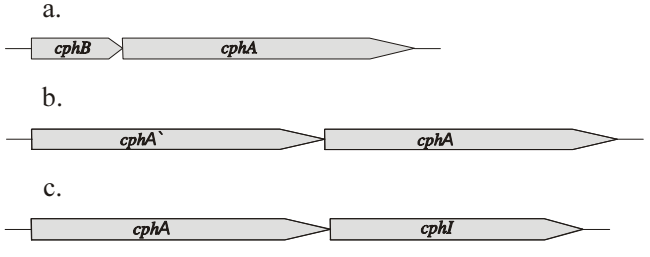

Fig. 3a–c Organization of *cph* genes in bacteria. **a** The organization found in **a** cyanobacteria, *Clostridium botulinum* strain ATCC 3802, and *Desulfitobacterium hafniense* strain DCB-2; **b** in *Bordetella bronchiseptica* strain RB50, *Bordetella pertussis* strain Tohama I, *Bordetella parapertussis* strain 12822, and *Nitrosomonas europaea* strain ATCC 25978; and **c** in *Acinetobacter* sp. strain ADP1

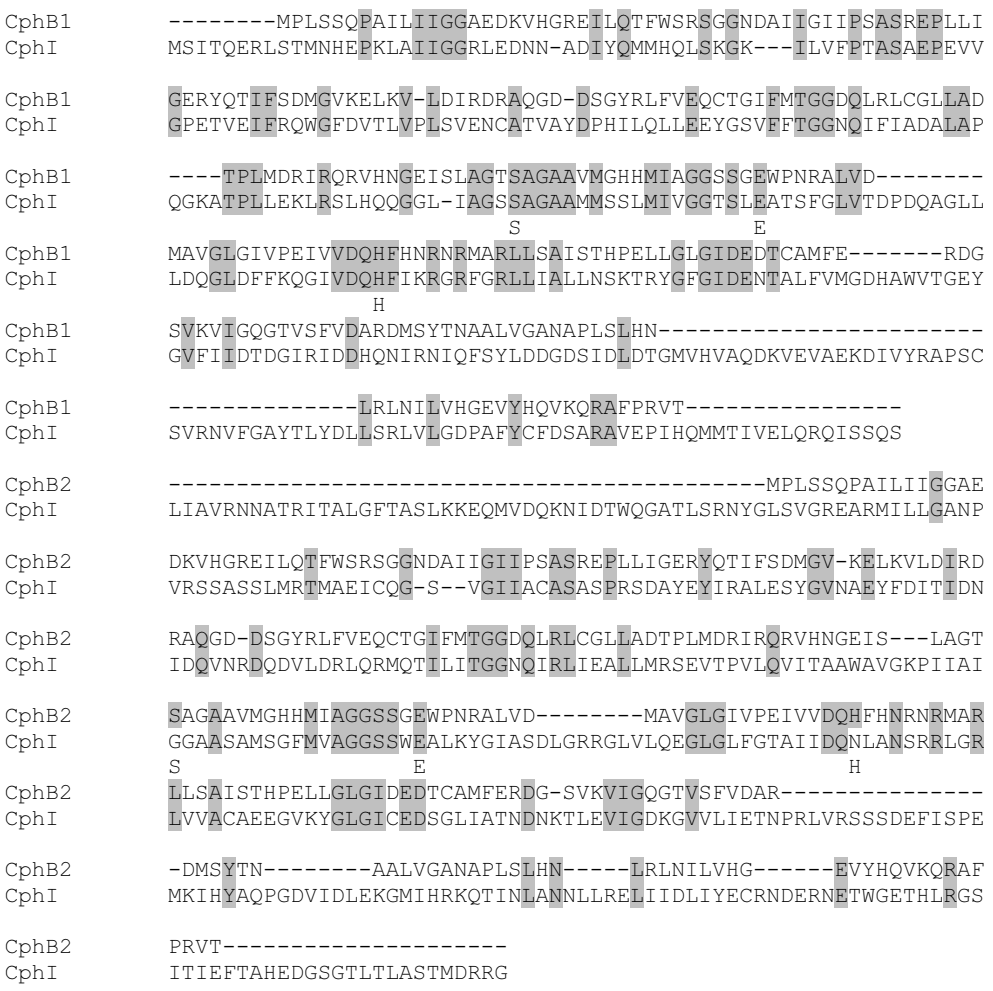

Fig. 4 Alignment of CphI (*Acinetobacter* sp. strain ADP1) and CphB (*Synechocystis* sp. strain PCC 6308). Identical amino acid residues are *shaded*. Amino acid residues of the catalytic triad proposed by Richter et al. (1999) are indicated below the sequences

Heterologous expression of *cphA* from *Acinetobacter* sp. DSM 587 in *E. coli* and comparison of the enzyme to recombinant CphA from the cyanobacterium *Synechocystis* sp. strain PCC 6308

To verify whether *Acinetobacter* sp. strain DSM 587 CphA is functionally active, *cphA* was amplified by PCR, ligated to pBluescript SK^{-} and expressed in *E. coli*. Freshly transformed cells of *E. coli* top 10 harboring the resulting plasmid with the *cphA* from *Acinetobacter* sp. strain DSM 587 inserted collinear to the *lac* promoter-operator region (pKOS1) formed colonies more whitish and opaque than those of strains not carrying the plasmid. Examination of the transformants by light microscopy revealed highly refractile cytoplasmic inclusions. Similar characteristics were previously also observed in strains of *E. coli* expressing cyanobacterial *cphA* (Aboulmagd et al. 2001). However, the ability to form whitish colonies was not stable and was lost after a few subcultivations, resulting in the occurrence of translucent or sectored colonies. Transformation of *E. coli* DH1 with pKOS1 resulted in more stable strains. In crude protein extracts obtained from cells of *E. coli* DH1 (harboring pKOS1) that were cultivated in Luria-Bertani medium, cyanophycin synthetase activity was detected. Probably due to the instability of the expression of the enzyme, the specific activities varied between 6.7 and 60.7% of the activity measured in crude extracts prepared from cells harboring a hybrid plasmid containing *cphA* from *Synechocystis* sp. strain PCC 6308 (Table 3).

Table 3 Comparison of cyanophycin synthetases from *Synechocystis* sp. strain PCC 6308 and *Acinetobacter* sp. strain DSM 587 with respect to substrate specificity and heterologous expression in *E. coli* DH1. Lysine incorporation was measured with lysine instead of arginine in the reaction mixture and is given as per cent of the activity obtained with arginine. *nd* Not detectable

	Cyanophycin content (%of dry weight)	Lysine content of the polymer (%)	Specific activity (%)	Lysine incorporation (%)
pBluescript SK^{-}	<0.2	–	<0.1	–
pSK^{-}::*cphA* (PCC 6308)	25.6	ca.10%	100	15
pSK^{-}::*cphA* (DSM 587)	1.8–7.5	nd	6.7–60.7	0.02

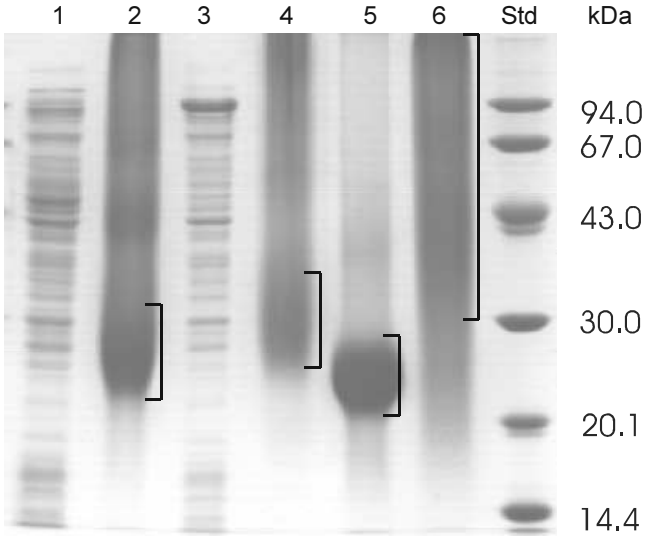

Fig. 5 Heterologous expression of cyanophycin synthetase and cyanophycin from different sources. Samples were separated in SDS-polyacrylamide (11.5% w/v) gels and stained with Serva Blue R. *Lane 1* Soluble fraction from *E. coli* DH1 harboring pSK⁻::*cphA* (PCC 6803) (35 μg), *lane 2* cyanophycin isolated from *E. coli* DH1 harboring pSK⁻::*cphA* (PCC 6803) (40 μg), *lane 3* soluble fraction from *E. coli* DH1 harboring pSK⁻::*cphA* (DSM 587) (35 μg), *lane 4* cyanophycin isolated from *E. coli* DH1 harboring pSK⁻::*cphA* (DSM 587) (40 μg), *lane 5* cyanophycin isolated from *Acinetobacter* sp. strain DSM 587 grown under phosphate-limited conditions for 33 h (40 μg), *lane 6* cyanophycin isolated from *Synechocystis* sp. strain PCC 6308 (40 μg). The molecular mass range of the main portion of the cyanophycin is indicated by brackets. The molecular mass standard proteins (*Std*) are given in kDa

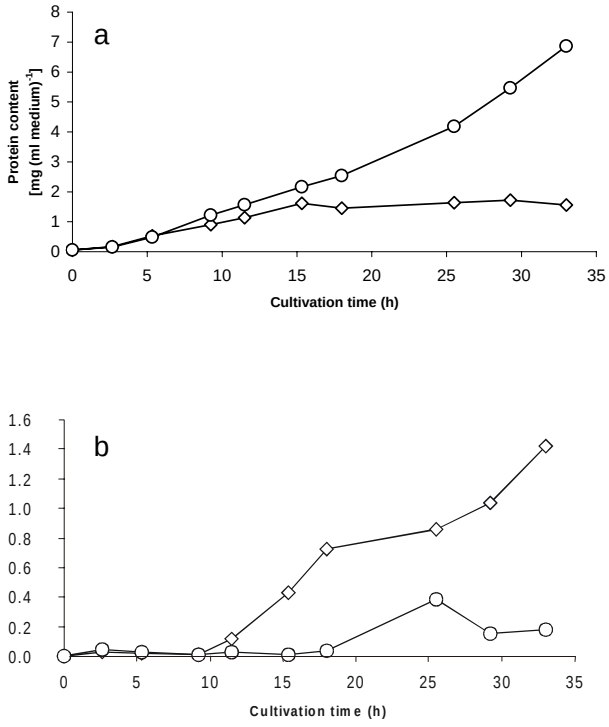

Fig. 6a, b Accumulation of cyanophycin by *Acinetobacter* sp. strain DSM 587. The strain was cultivated in Tris-buffered medium containing 1.3 mM (❍) or 64 μM (❑) phosphate. **a** Total protein content as determined by the method of Schmidt et al. (1963); **b** cyanophycin content in % of total protein

A polymeric material with solubility at different pH values similar to those of cyanophycin isolated from cyanobacteria or recombinant *E. coli* expressing cyanobacterial CphA, was isolated from *E. coli* DH1 harboring pKOS1. In accordance with the variations of the specific activities of cyanophycin synthetases, the content of this polymeric material also varied considerably, between 1.8 and 7.5% of the cellular dry matter (Table 3).

HPLC analysis of the hydrolyzed polymer revealed that the product of the cyanobacterial enzyme contained not only arginine and aspartic acid, but in addition about 10% lysine. In contrast, the material isolated from *E. coli* DH1 expressing *cphA* from *Acinetobacter* did not contain any detectable lysine; it consisted exclusively of aspartic acid and arginine in equimolar amounts. With lysine instead of arginine in the radiometric enzyme assay using the crude extract, the cyanobacterial CphA was shown to incorporate lysine into the polymer at 15% of the rate obtained with arginine. In contrast, for CphA from *Acinetobacter* sp. strain DSM 587, only 0.02% lysine incorporation was measured (Table 3). As for CphA from *Synechocystis* sp. strain PCC 6308, activity strongly depended on the presence of ATP, Mg^{2+}, a cyanophycin primer and both arginine and aspartic acid in the reaction mixture. Without ATP, arginine or Mg^{2+} in the reaction mixture, the activity of the enzyme was 0.3% or less of the activity in the complete reaction mixture. Without aspartate or a cyanophycin primer, the activity was 1.0% and 1.2%, respectively. The apparent molecular mass of the isolated polymer from both recombinant strains as estimated by SDS-PAGE was between 25 and 40 kDa (Fig. 5).

Accumulation of cyanophycin in *Acinetobacter* sp. DSM 587 and characterization of the isolated material

After having demonstrated that *cphA* from *Acinetobacter* sp. DSM 587 encoded a functionally active enzyme in *E. coli*, we were interested to see whether the natural host also synthesized cyanophycin. The accumulation of cyanophycin by *Acinetobacter* sp. strain DSM 587 was strongly dependent on the phosphate concentration in the medium and on the growth phase. Under phosphate-limited conditions, the strain accumulated up to 14 μg cyanophycin per mg protein after 33 h cultivation. Cyanophycin accumulation occurred almost exclusively in the stationary phase. After cultivation without phosphate limitation, the cyanophycin content was only 1.8 μg per mg protein. Cyanophycin accumulation started in the late-exponential growth phase and continued throughout the stationary phase (Fig. 6). HPLC analysis of the isolated material revealed that *Acinetobacter* sp. strain DSM 587 accumulated a cyanophycin consisting of equimolar amounts of aspartic acid

and arginine as sole constituents. The apparent molecular mass of the material as determined by SDS-PAGE ranged between approximately 21 and 28 kDa (Fig. 5). The solubility of the material was identical to that of cyanophycin isolated from the cyanobacterium *Synechocystis* sp. strain PCC 6308 or from *E. coli* DH1 expressing *cphA* of *Acinetobacter* sp. strain DSM 587, being soluble in 0.1 M HCl and 0.1 M NaOH and insoluble at neutral pH. Specific cyanophycin synthetase activity in phosphate-limited cells of *Acinetobacter* sp. DSM 587 was shown to be 0.007 U per mg protein.

Discussion

Analysis of available bacterial genome sequences identified ORFs putatively encoding proteins of high similarity to known cyanophycin synthetases from cyanobacteria in several strains of bacteria belonging to different phylogenetic taxa and not closely related to these oxygenic photosynthetic bacteria. In addition, these bacteria also display diverse types of metabolism, including aerobic respiration, anaerobic fermentation, anaerobic respiration and chemolithoautotrophy. Therefore, the capability for cyanophycion biosynthesis is not restricted to only cyanobacteria but also occurs among other groups of bacteria.

At least in one of these strains, the aerobic *Acinetobacter* sp. strain DSM 587, cyanophycin accumulation under phosphate-limited conditions could be shown. Heterologous expression of *chp*A from this strain in *E. coli* also showed the corresponding CphA to be functionally active, leading to accumulation of cyanophycin in *E. coli*. Cyanophycin has been proposed to function as a dynamic nitrogen buffer during nitrogen fixation in cyanobacteria (Carr 1988). Since nitrogen fixation does not occur in *Acinetobacter* sp., the function of cyanophycin in this strain appears to be that of a nitrogen and carbon storage polymer. This view is supported by the accumulation of cyanophycin under phosphate-limited conditions. Since *Acinetobacter* spp. also have the ability to accumulate other carbon storage compounds such as lipids and polyhydroxyalkanoates in large quantities (Alvarez et al. 1997; Aldor and Keasling 2001), the relatively small quantities of cyanophycin accumulated are probably more important as a nitrogen rather than as a carbon storage compound.

The major differences between cyanophycin from *Acinetobacter* sp. strain DSM 587 and from *Synechocystis* sp. strain PCC 6308 are in its molecular mass in the original strains and in its composition when produced heterologously in *E. coli*. Although cyanophycin from *Synechocystis* sp. strain PCC 6308 is composed solely of aspartic acid and arginine, the cyanophycin synthetase incorporates also lysine into the polymer in vitro and when expressed in *E. coli*. In contrast, cyanophycin synthetase from *Acinetobacter* sp. strain DSM 587 did not incorporate lysine into the polymer to any detectable extent. The apparent molecular mass of cyanophycin from *Acinetobacter* sp. strain DSM 587 has a relatively narrow range, between 21 and 28 kDa. Similarly narrow ranges were also revealed for cyanophycin produced heterologously in *E. coli* employing several cyanobacterial *cphA* genes (Ziegler et al. 1998; Oppermann-Sanio et al. 1999). In cyanobacteria, the apparent molecular masses range widely, between 25 and 125 kDa.

Although no function has yet been assigned to CphI, it appears very likely based on the similarities to CphB and the location of the gene that it is involved in cyanophycin metabolism. The same is likely to be true for the putative CphB homologues in the gram-positive bacteria. Interestingly, no putative ORFs encoding proteins with substantial similarity to CphB have been detected in the β-Proteobacteria. If the *cphA* genes are actively expressed in these bacteria and do not merely represent silent genes, some other cyanophycin-degrading enzymes must be present.

The occurrence of *cphA′* in the genomes of some of these bacteria is also intriguing. One possible function could be that cyanophycin synthetase proteins in these bacteria do not represent homodimers as in cyanobacteria (Ziegler et al. 1998; Aboulmagd et al. 2001), but heterodimers with CphA′ and CphA as constituents. Interestingly, major differences between CphA′ and CphA occurred in amino acid residues supposedly involved in both ATP-binding sites, notably the J-loop of the ATP-grasp and the GXXGKT/S motif also conserved in ligases involved in murein biosynthesis. These sites are also considered to bind ATP in cyanophycin synthetases (Ziegler et al. 1998; Berg et al. 2000). Lacking the ability to bind ATP, another function of CphA′ may even be that of a cyanophycinase. Cloning and heterologous expression of *cphA′* will reveal insights into the function of CphA′.

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