Isolation of cyanophycin degrading bacteria – Cloning and characterization of an extracellular cyanophycinase gene (cphE) from Pseudomonas anguilliseptica strain BI

THE CPHE GENE FROM PSEUDOMONAS ANGUILLISEPTICA BI ENCODES A CYANOPHYCIN (CGP)-HYDROLYZING ENZYME

Martin Obst*§, Fred Bernd Oppermann-Sanio#§, Heinrich Luftmann*¶, and Alexander Steinbüchel‡§

From the §Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Corrensstraße 3, D-48149 Münster, Germany, and the ¶Institut für Organische Chemie, Westfälische Wilhelms-Universität Münster, Corrensstraße 40, D-48149 Münster, Germany

* Tel.: 49 251 833 9810; E-mail: obstimar@uni-muenster.de
# Tel.: 49 251 833 3980; E-mail: opperma@uni-muenster.de
+ Tel.: 49 251 833 3299; E-mail: luftman@uni-muenster.de
‡ To whom correspondence should be addressed. Tel.: 49 251 833 9821;
Fax: 49 251 833 8388; E-mail: steinbu@uni-muenster.de
Cloning of the CGPase Gene from Pseudomonas anguilliseptica BI

Summary

Eleven bacteria capable of utilizing cyanophycin (cyanophycin granule polypeptide, CGP) as carbon source for growth were isolated. One isolate was taxonomically affiliated as Pseudomonas anguilliseptica strain BI, and the extracellular cyanophycinase (CphE) was studied because utilization of cyanophycin as carbon source and extracellular cyanophycinases were hitherto not described. CphE was detected in supernatants of CGP cultures and purified from a corresponding culture of strain BI employing chromatography on the anion exchange matrix Q-Sepharose and on an arginine-agarose affinity matrix. The mature form of the inducible enzyme consisted of one type of subunit with \( M_r \) 43 kDa and exhibited high specificity for CGP, whereas proteins and synthetic polyaspartic acid were not or only marginally hydrolyzed. Degradation products of the enzyme reaction were identified as aspartic acid-arginine dipeptides (\( \beta \)-asp-arg) by high performance liquid chromatography and electrospray ionization mass spectrometry. The corresponding gene (cphE; 1254 base pairs) was identified in subclones of a cosmid gene library of strain BI by heterologous active expression in Escherichia coli, and its nucleotide sequence was determined. The enzyme exhibited only 27-28% amino acid sequence identity to intracellular cyanophycinases (CphB) occurring in cyanobacteria. Analysis of the amino acid sequence of cphE revealed a putative catalytic triad consisting of the motif G-X-S-X-G plus a histidin and most probably a glutamate residue. In addition, strong inhibition of the enzyme by Pefabloc\textsuperscript{®} and PMSF indicated that the catalytic mechanism of CphE is related to that of serine type proteases. Quantitative analysis on the release of \( \beta \)-asp-arg dipeptides from carboxy-terminal labelled CGP gave evidence for an exo-degradation mechanism.

Introduction

Cyanophycin (cyanophycin granule polypeptide, CGP) is a natural occurring poly(amino acid), which is synthesized in most cyanobacteria as nitrogen, carbon and energy storage compound in the early stationary growth phase (1,2). The water insoluble CGP is accumulated intracellularly in the form of membraneless granules (3) and is degraded by the cells when growth is resumed. The backbone of this unique biopolymer consists of \( \alpha \)-amino-\( \alpha \)-carboxy-linked L-aspartic acid monomers. Most of the \( \beta \)-carboxylic groups are covalently bound to the \( \alpha \)-amino groups of L-arginine residues (4,5); in recombinant Escherichia coli
expressing cyanobacterial CGP synthesizing enzymes (see below) a significant fraction of arginine is replaced by lysine (6).

Whereas much information has been obtained concerning the non-ribosomal biosynthesis of CGP, which is catalysed by the cyanophycin synthetase (CphA, (7) and cited references therein), only few reports are available on the intracellular degradation of CGP. Intracellular CGP degradation was first observed in crude extracts of soluble proteins prepared from cells of *Anabaena cylindrica* (5). The corresponding enzyme, cyanophycinase (CphB), was purified from a recombinant *E. coli* harbouring the *cphB* gene from *Synechocystis* sp. PCC6803 and characterized in detail (8). Dipeptides consisting of arginine plus aspartic acid and free arginine were identified as products of CGP degradation in addition to small amounts of aspartic acid (8). In contrast to intracellular degradation, nothing is known about the extracellular decomposition of this biopolymer by bacteria or other microorganisms.

In this study, we demonstrate for the first time that CGP can be easily degraded and utilized as sole carbon source for growth by a variety of non-cyanobacterial eubacteria isolated from different habitats. Because it is known that CGP is resistant to a wide range of commercially available proteases (4,9), these bacteria must possess an enzyme specialized for CGP degradation. We report on the isolation of a strain of the species *Pseudomonas anguilliseptica*, describe the substrate utilization capabilities of this bacterium and the purification of an extracellular cyanophycinase (extracellular CGPase, CphE) from culture supernatant of cells grown on CGP. Furthermore, CphE was biochemically characterized in order to reveal the degradation mechanism and to identify the cleavage products. In addition, the CGPase gene (*cphE*) of the isolated *P. anguilliseptica* strain BI was cloned and characterized.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, and Growth of Bacteria*—CGP degrading bacteria isolated in this study are listed in Table 1. These strains were either grown on Standard I complex medium (MERCK, Darmstadt, Germany) or on basic inorganic medium B (10) for CGP degradation and substrate utilization experiments. The concentrations of CGP and other carbon sources added to the media are indicated in the text. All isolates were grown at 30°C. The following microorganisms were used as reference strains in substrate utilization assays on solid CGP-medium (see below) with 0.05% (w/v) glucose as additional carbon source: *Escherichia coli* K12 (wild type), *Pseudomonas putida* KT2440 (11), *Micrococcus luteus* (DSMZ 348), *Bacillus subtilis* 168°
For CGP production an *E. coli* DH1 strain harbouring plasmid pMa/c5-914::cphA expressing *cphA* from *Synechocystis* PCC6803 (12) was employed (see below). *E. coli* strains were usually grown at 37 °C in Luria-Bertani (LB) medium or terrific broth (TB) complex medium (13).

**Preparation of Selective Media for the Detection and Isolation of CGP Degrading Microorganisms**—Samples from different sources were spread on solid basic inorganic medium B (10) supplemented with trace element solution SL 7 (14) and overlaid with 0.5% (w/v) agar containing 0.2% (w/v) of CGP. For the preparation of the overlay agar diethylether-sterilized CGP was first dissolved in 0.1 N HCl and then added to sterile medium under vigorous stirring to avoid the formation of inhomogenous CGP precipitates. For adjustment of the pH value an equal volume of 0.1 N NaOH was added before pouring plates.

**Isolation, Manipulation, and Analysis of DNA**—For isolation of plasmid DNA the lithium preparation method was applied (15). Total genomic DNA of *P. anguilliseptica* strain BI was isolated according to the method of RAO et al. (16). After partial digestion with the endonuclease *Pst*I, genomic DNA fragments were ligated to the cosmid vector pHC79 (17), and *E. coli* strain S17-1 (18) was used as a recipient for transduction of the cosmid library. A Gigapack® III XL packaging extract (Stratagene, La Jolla, California, USA) was employed for the packaging of DNA and subsequent infection of strain S17-1 as described by the manufacturer. *E. coli* strain XL1-Blue (Stratagene, La Jolla, California, USA) was used in combination with pBluescript SK+ (Stratagene, La Jolla, California, USA) for cloning of a *Pst*I restriction subfragment (2600 bp), sequence analysis of *cphE* and for heterologous production of the enzyme.

5´-IRD 800-labelled synthetic oligonucleotides (MWG-Biotech, Ebersberg, Germany) were used as primers, and a Sequi Therm EXCEL TM II long-read cycle sequencing kit (Epicentre Technologies, WI, USA) was employed for DNA-sequencing according to the “primer-hopping strategy” (19). Analysis was done in 6% (w/v) acrylamide gels using Sequagel XR® (acylamide/urea), Complete® (buffer reagent) solutions (National Diagnostics, Simmerville, USA) and buffer containing 89 mM TRIS, 89 mM boric acid and 2 mM EDTA in a LI-COR 4000L automatic sequencing apparatus (MWG-Biotech, Ebersberg, Germany). Nucleic acid sequence data and deduced amino acid sequences were analyzed with the sequence analysis software CAP (Contig Assembly Programm; (20)), ClustalX 1.8 (21) and Genamics Expression 1.1.

The 16S rRNA gene was amplified from total DNA (see above) using oligonucleotide primers as described before (22). After purification of the PCR products with a NucleoTrap® CR kit (Macherey-Nagel, Düren, Germany), their nucleotide sequences were determined as described above. The 16S rDNA sequence
was aligned with published sequences from representative *Pseudomonas* species from the National Centre for Biotechnology Information (NCBI) database.

*Nucleotide Sequence Accession Numbers*—The nucleotide and amino acid sequence data reported here for *cphE* have been submitted to the NCBI database under accession number AY065671. The 16S rRNA gene sequence data of *P. anguilliseptica* strain BI were deposited in the NCBI database under accession number AF439803.

*Cyanophycin Production*—For production of native CGP, *Synechocystis* sp. strain PCC6308 was cultivated in full-strength BG11 medium (23) in an 80 l closed tubular glass photobioreactor as described before (24). Also, a recombinant *E. coli* DH1 harbouring plasmid pMa/c5-914::cphA (see above) with a temperature sensitive inducible promotor was employed for the production of CGP. A 42 l Biostat UD30 stainless steel bioreactor (B. Braun Biotech International, Melsungen, Germany) with TB complex medium was used for production as described previously (12).

*Isolation of Cyanophycin*—After cell harvest CGP was isolated according to the method of Simon (25), which was modified by applying only one washing step at each Triton X100®-concentration and two additional centrifugation steps of the acidic and neutralized suspensions, respectively. The purity of CGP was controlled both by SDS-PAGE with subsequent Coomassie staining (26) and by HPLC analysis after acid hydrolysis of the polymer and subsequent derivatization of amino groups with OPA (o-phtaldialdehyde) reagent (27).

*Taxonomic Determination with Physiological Tests*—Motility and Gram behaviour were determined as described before. Oxidase (Bactident® Oxidase test strips from Merck, Darmstadt, Germany) and catalase tests were performed according to standard protocols. Further determinations were done by using the API20NE test kit (BioMérieux, Marcy-l’Etoile, France).

*Analytical Methods*—Reversed phase HPLC was used to determine the products of enzymatic CGP degradation as described by the method for the quantitative determination of amino acids (27). Electrospray ionization mass spectrometry (ESI) was applied for identification of the final degradation product of CGP by mass determination and structural analysis (28). All measurements were performed employing a Quattro LCZ system (Micromass, Manchester, UK) with a nanospray inlet.

*Purification of the Extracellular Cyanophycinase from P. anguilliseptica Strain BI*—A cell free supernatant from a CGP culture was obtained by sedimentation of the cells in the late exponential growth phase by centrifugation and subsequent filtration of the supernatant through a 0.2 µm nitrocellulose membrane. All
steps were carried out at 4°C and in the presence of 50 mM sodium phosphate buffer (pH 8.3). Further components added to the buffer are mentioned below. After concentration in an ultrafiltration chamber (Amicon, Beverly, MA, USA) using a YM10 membrane, the buffered solution was applied onto a MonoQ HR5/5 anion exchange column (Amersham Pharmacia LKB Biotechnology, Uppsala, Sweden). After washing the column with 2 bed volumes [BV] of buffer, CGPase was eluted with a linear NaCl-gradient (0-1M) employing an increase of NaCl-concentration of 17 mM/ml and a total flow rate of 1 ml/min. Active fractions (1 ml) were detected after transfer of 10 µl of the respective eluates onto CGP-overlay plates (see above) by the occurrence of halos after 5-40 min of incubation at 30°C. Fractions with high activity were combined, desalted by ultrafiltration (see above), and applied onto an arginine-agarose column (5 ml BV; Sigma Chemical, St. Louis, USA). For selective elution of the enzyme, an arginine gradient (0-1 M) was applied. To avoid nonspecific protein binding and to prevent the enzyme from binding in the presence of high arginine concentrations, the buffer contained in addition 100 mM NaCl.

SDS-PAGE of active enzyme fractions or CGP samples was performed in 11.5% polyacrylamide gels according to standard protocols (29). Proteins were visualized by the Coomassie staining method (26). An “in-gel” renaturation method described for activity staining with proteases after SDS-PAGE (30) was used to obtain reactivated CGPase after separation of enzyme subunits according to their apparent molecular mass under denaturing conditions. The ability of reactivated CGPase to form degradation halos was tested by the application of a thin CGP-agar layer (see above) on top of buffer-pretreated gels. Protein concentrations were determined by the procedure of Bradford (31).

**Characterization of the Purified CGPase**--For determination of the substrate specificity of the CGPase the purified recombinant enzyme was incubated at 30°C in 1 ml of 50 mM sodium phosphate buffer (pH 8.3) with various polypeptide substrates. Each reaction contained 1 mg of the respective substrate and 1.6 µg of enzyme. The reaction was stopped after 120 minutes by incubation at 70°C for 5 min. After centrifugation, 100 µl aliquots of supernatant were incubated at 95°C for 5 min in the presence of 1.25% ninhydrin (Merck, Darmstadt, Germany) in 1 ml of total reaction volume. Subsequently they were assayed photometrically at 570 nm for the presence of released hydrolysis products. Bovine casein (Hammersten-grade) was from Merck (Darmstadt, Germany), bovine serum albumin (BSA) from Roth (Karlsruhe, Germany), and poly(α,β-D/L-aspartic acid) (*M*ₐ = 11,000) was obtained from Bayer (Leverkusen, Germany). Labelling experiments were performed by enzymatic elongation of the carboxyl-terminus of a CGP primer (32). L-[U-¹⁴C]-labelled arginine was incorporated into the polymer chain using purified cyanophycin synthetase from *Synechocystis*
sp. strain PCC6308 heterologously produced in *E. coli* (27). Labelled CGP was incubated at 30°C with 1.6 µg of CGPase in 50 mM sodium phosphate buffer (pH 8.3) under vigorous shaking. For heat inactivation of the enzyme 50 µl samples were transferred to test tubes containing 500 µl of water preincubated at 70°C. After 5 minutes of inactivation, the samples were transferred onto ice for 5 minutes to allow for CGP reprecipitation. After subsequent centrifugation, 50 µl of supernatant were mixed with 500 µl Hydroluma® Scintillation cocktail (J.T. Baker, Deventer, Netherlands). Radioactivity was measured with a model LS 6500 scintillation counter (Beckman Instruments, München, Germany; (27)).

RESULTS

*Enrichment and Isolation of CGP Degrading Bacteria*—To screen for CGP degrading bacteria, samples from typical habitats of CGP producing cyanobacteria were plated onto solid mineral medium containing CGP as sole carbon source. Due to the insolubility of CGP at neutral pH, the agar was turbid. Colonies of CGP degrading microorganisms were recognized due to the formation of degradation halos which appeared after 12–18 h of incubation at 30°C (Fig 1A). Based on this feature, axenic cultures of nine bacterial strains were finally isolated from Baltic sea water, different pond sediments and sewage sludge (Table 1). In addition to the newly isolated CGP degrading strains, other bacteria from our culture collection were also tested, and two additional strains with CGP degradation capability were detected (strains BE2 and PAS1, Table 1). However, *E. coli* K12, *P. putida* KT2440, *M. luteus*, *B. subtilis 168* and *B. megaterium* were not able to cause formation of halos on CGP-overlay agar plates, although some of these bacteria (e.g. *B. subtilis*) are known to use proteins as nutrients.

*Table 1*

*Characterization of CGP Degrading Bacteria through Substrate Utilization Patterns and Taxonomic Classification.*—All isolates tested (from Baltic sea water, pond sediment and sewage sludge, i.e. "A-, B- and D-series"; see Table 1) were Gram-negative, oxidase- and catalase-positive rod shaped bacteria. With the exception of isolate DXIII all strains showed motility. Applying the API 20NE test kit, two isolates (DIII and DIV) revealed acceptable identification profiles. Both strains were taxonomically affiliated as strains of the species *Pseudomonas alcaligenes* (Table 2). As listed in Table 1, most strains isolated in this study showed growth on the amino acid constituents of CGP, i.e. on aspartic acid and arginine. For most strains growth with
arginine was faster than with aspartic acid. Only isolate BI showed no growth on aspartic acid. Strain PAS 1 from the culture collection of our institute also did not grow on aspartic acid as sole carbon source. None of the isolates was able to grow on synthetic poly(α,β-D/L-aspartic acid) (data not shown). With poly(γ-D-glutamic acid) (PGA) as sole carbon source, only isolate DXIII and to some extend also *P. alcaligenes* strain BE2 (33) showed growth (Table 1). On bovine serum albumin as sole carbon source, only strain PAS I and *P. alcaligenes* strain BE2 exhibited good or poor growth, respectively. Citrulline and ornithine, two putative degradation products of arginine, were not utilized as carbon sources for growth by any of the bacteria investigated in this study. The only exceptions were isolate AVN, which was able to grow on ornithine, and *B. subtilis* 168*, which utilized citrulline (data not shown).

-Table 2-

**Taxonomic Classification of Isolate BI by 16S-rRNA Gene (rDNA) Sequence Analysis.**—For several reasons, isolate BI was a good candidate for a more detailed investigation of CGP degradation. Therefore, the taxonomic position of the isolate was determined. Analysis of the 16S-rDNA sequence of isolate BI revealed 98% identity to the nucleotide sequence of all three *Pseudomonas anguilliseptica* strains available at the NCBI data base including the *P. anguilliseptica* type strain NCIMB 1949. Maximum sequence identity to other species of the genus *Pseudomonas* was only in the range of 95-96% (see also Fig. 2). Therefore, the new isolate was referred to as *Pseudomonas anguilliseptica* strain BI.

Preliminary 16S-rDNA sequence data of strain PAS 1 (about 1000 bp) revealed that this strain most probably belongs to the genus *Streptomyces*. This finding corresponds well with the streptomycete like habitus of this strain, e.g. the formation of exospores in ageing colonies.

-Fig.1-Fig.2-

**Growth Kinetics of P. anguilliseptica** strain BI.—The ability of *P. anguilliseptica* strain BI to grow on CGP as the sole carbon source was investigated in more detail. Therefore, growth of this strain on CGP and on its amino acid constituents as well as on the non related substrate citrate was monitored over 24 hours (Fig. 3). Living cell counts for the cyanophycin culture revealed that growth of the cells started at about 4 h of incubation (data not shown) after inoculation from a citrate culture. The turbidity caused by suspended CGP particles disappeared visibly during incubation. Strain BI grew best with a combination of arginine and
aspartic acid if these amino acids were provided at a molar ratio according to their proportional masses in the CGP molecule (248 Klett units, KU). Growth on CGP led to a maximum optical density of 202 KU which is in the range of the optical density (OD) of the citrate culture. Slightly weaker growth was detected for the arginine culture (182 KU). No increase or change of the OD occurred in the control (sterile medium containing citrate) or in mineral salts medium containing aspartic acid as sole carbon source (Fig. 3). During growth on CGP 46% (w/w) of the polymer were converted into cellular dry matter by *P. anguilliseptica* strain BI.

Purification of the Extracellular CGPase from *P. anguilliseptica* Strain BI—The extracellular CGPase of *P. anguilliseptica* was purified to electrophoretic homogeneity from CGP-grown cultures by application of anion exchange chromatography on Q-Sepharose followed by L-arginine-agarose affinity chromatography (Fig.1C). The latter is usually used for different purposes, e.g. purification of transfer RNA molecules (35). The L-arginine-agarose matrix was highly specific for the binding of CGPase under the employed conditions, revealing a high affinity of the enzyme to this matrix. Therefore, an arginine gradient (0-1 M) in sodium phosphate buffer was applied for the elution of the CGPase. To further reduce nonspecific binding of other proteins, the sodium phosphate buffer additionally contained 100 mM NaCl. In the absence of NaCl, arginine had to be applied at concentrations higher than 3 M for total release of the enzyme from the affinity matrix, again reflecting the high affinity of the CGPase to the arginine residues coupled to the matrix. During enzyme purification active fractions were detected by their ability to cause rapid halo formation on CGP-overlay agar plates (Fig. 1B). These halos occurred mostly within a few minutes to 2 hours, and their diameters indicated the activity of the enzyme in the sample.

SDS-polyacrylamide gelelectrophoresis revealed an apparent molecular mass of the subunits of the enzyme of 43 kDa (Fig. 1C). By employment of an “in-gel” renaturation method, previously used for the detection of proteases in SDS-polyacrylamide gels (30), in combination with subsequent application of a thin CGP-agar layer on top of the “renaturated” gel, it was possible to detect reactivated CGPase by the formation of a degradation halo at the position corresponding to a protein of the expected molecular mass of 43 kDa (Fig. 1D). This finding clearly proved that the 43 kDa protein represented the subunit of the CGPase and that the enzyme consisted of only one type of subunit or of subunits with identical apparent molecular masses.
Purification of the *P. anguilliseptica* CGPase from a Recombinant Strain of *E. coli*—The heterologously produced enzyme was purified in the same way from the soluble fraction of *E. coli* cells harbouring pBluescript SK−::cphE (construction see below) grown in TB medium. The enzyme was not excreted from the cells when it was produced by *E. coli*. In culture supernatants enzyme activity was not detectable. In addition, halo formation on CGP-overlay LB agar plates occurred only after 3 days of incubation, thus indicating that the release of the enzyme presumably occurred due to partial cell lysis during ageing of the cells. By this purification method, the activity of the heterologously expressed enzyme was enriched 15-fold, and 49% of the total activity were recovered as confirmed by calculation of halo forming units in a plate diffusion assay using purified enzyme as a standard (data not shown).

Cloning and Analysis of the Extracellular CGPase Gene from *P. anguilliseptica* BI—Applying the restriction endonuclease *Pst*I for partial digestion of genomic DNA from *P. anguilliseptica* BI, fragments with a broad size range were obtained and subsequently ligated to the cosmid vector pH79C. After transduction of the cosmid library into *E. coli* S17-1, approximately 3000 tetracycline resistant clones were obtained and tested for their ability to degrade CGP by transfer onto LB agar plates overlaid with a thin layer of CGP containing medium. After 3 days of incubation at 37°C, one colony was detected which was surrounded by a halo indicating degradation of the polymer in the CGP layer. The plasmid containing the CGPase encoding genomic fragment from *P. anguilliseptica* BI (26 kbp) was isolated from the respective clone. Twelve subfragments were obtained after total digestion with *Pst*I and ligated to the vector pBluescript SK−. Subsequent transformation of *E. coli* XL1-Blue with the resulting mixture of pBluescript SK− construction products led to the identification of a bacterial colony capable of forming halos on a CGP-overlay plate.

Biochemical Characterization of the CGPase—*P. anguilliseptica* strain BI produced the extracellular CGPase only when CGP was present in the medium. In complex standard 1 medium no enzyme activity was detected. As substrate utilization patterns of strain BI indicated (Table 1), no correlation occurred between the ability to degrade CGP and the utilization of other polyamide substrates, including BSA, poly(γ-D-glutamic acid) or synthetic poly(αβ-D/L-aspartic acid) (data not shown). This indicated that the CGPase is not employed by the bacterium for nonspecific hydrolysis of polyamide substrates. This was also confirmed by studies on the substrate specificity of the purified enzyme. Using the enzyme purified from the recombinant strain of *E. coli* (see above), the release of degradation products from the polyamide substrates CGP, BSA,
bovine casein and poly(α,β-D/L-aspartic acid) was investigated employing ninhydrin reagent for detection of released amino groups (Fig. 4). After two hours of incubation in presence of purified enzyme, only CGP samples showed a significant release of ninhydrin positive degradation product, whereas BSA and bovine casein samples revealed only a very weak release of reactive products amounting to 3.9 or 6.4% of that obtained with CGP, respectively (Fig. 4). From synthetic poly(α,β-D/L-aspartic acid) no release of ninhydrin positive material was detected (Fig. 4).

Using purified recombinant CGPase, degradation of cyanobacterial CGP (Synechocystis PCC6308) was visualized by SDS-PAGE and subsequent nonspecific protein staining (Fig. 5). Degradation of the high molecular weight polydisperse biopolymer (about 43–100 kDa; lane 2) to low molecular weight material (lanes 3-9) is demonstrated in Fig. 5. The lack of detectable high molecular weight material after 160 minutes of incubation (lane 10) corresponded well with the nearly exclusive detection of the final degradation product of CGP (see HPLC and ESI analysis below) indicating total degradation of CGP having occurred within that time. A splitting of the initial molecule population (lane 2) into two populations of molecules exhibiting two different molecular weight ranges was observed during the incubation (Fig. 5, lanes 3-9).

The composition of the amino acid constituents of CGPs varies depending on the source of CGP: cyanobacterial CGP isolated from Synechocystis PCC6308 contains only aspartic acid plus arginine, whereas in CGP isolated from cells of a recombinant E. coli expressing the PCC6803 cyanophycin synthetase gene some arginine residues were replaced by lysine residues (6). The final degradation products of the enzyme reaction on both CGPs were determined. As shown in Fig. 6 degradation of the cyanobacterial CGP led to the formation of only one detectable degradation product after separation in HPLC, whereas recombinant polymer produced by E. coli led to the formation of two main products. Therefore, it seemed likely that dipeptides of β-asp-arg or of β-asp-arg plus β-asp-lys were formed during the degradation process, respectively. A degradation mechanism producing oligomers would have led to the formation of more than two different products in case of the recombinant CGP, resulting most probably in the appearance of more
than two peaks in the HPLC chromatogram. Final proof for the presence of the dipeptide was obtained by application of electrospray ionization mass spectrometry (ESI) on the isolated degradation product of cyanobacterial CGP (see above). The degradation products gave a strong signal in the positive ion ESI/MS spectrum at m/z 290 ([M+H]+, Fig. 7A) corresponding to a molecular weight of 289 Da, as it is calculated for the β-asn-arg dipeptide. Furthermore, analysis of the m/z 290 molecule by ESI/MS/MS revealed a fragmentation pattern characteristic for the structure of the β-asn-arg dipeptide (Fig. 7 B). The peaks at m/z 175 and m/z 116 represent the molecules occurring after fragmentation of the dipeptide at the β-amide bond. This is indicated by the –115 arrow pointing at m/z 175 ([M + H – HO₂C-CHNH₂-CH=O=C=O]⁺) and the –174 arrow pointing at m/z 116 ([HO₂C-CHNH₂-CH=O + H]⁺) respectively (see Fig. 7 B).

To elucidate the mechanism of CGP degradation, carboxy-terminal L-[U-¹⁴C]-arginyllabelled CGP was synthesized using the cyanophycin synthetase from Synechocystis PCC6308. Incubation of labelled CGP with recombinant CGPase resulted in an immediate release of radioactivity that continued for approximately 7 minutes (see Fig. 8). Together with the observation that after addition of CGPase the release of β-asn-arg dipeptides was detectable by HPLC analysis within 30 seconds (data not shown), an exo-degradation mechanism proceeding (at least partially) from the carboxyl-terminus of CGP seems to be most likely for the enzyme reaction.

Inhibitor studies showed that the extracellular CGPase of P. anguilliseptica B1 is strongly inhibited by the serine protease inhibitors PMSF and Pefabloc® (63% and 92% respectively; see Table 3). Inhibition of the enzyme by Pefabloc® was additionally confirmed by the inhibition of halo formation (Table 3). CGPase activity was only slightly (and inhibitor concentration independent) decreased by the thiol protease inhibitor leupeptin (13%). The observed decrease in activity in this cases is most probably due to interference with the “OPA-derivatization” method necessary for HPLC analysis. Application of the metalloprotease inhibitor EDTA led to a strong reduction of the release of detectable β-asn-arg dipeptides in the enzyme reaction (Table 3). This was most probably due to the formation of precipitates occurring during derivatization. The ability of the enzyme to form degradation halos in CGP-overlay agar in the presence of 30-500 mM EDTA was, however, not affected. Only the tryptophan oxidant N-bromosuccinimide totally prevented the release of β-asn-arg and the formation of degradation halos on CGP-overlay plates. The latter occurred after 30 minutes of incubation together with CGPase, even if N-bromosuccinimide was applied at concentrations ≥1 mM. Therefore, a tryptophan residue may be involved in the process of CGP degradation (compare Fig. 9).
Molecular Characterization of the Extracellular CGPase Gene (CphE) from *P. anguilliseptica*—The coding gene for the extracellular CGPase (CphE) from *P. anguilliseptica* strain BI was identified by its heterologous active expression from a gene library of total DNA in *E. coli* S17-1 (see above). One *E. coli* clone harbouring a 26-kbp fragment of *P. anguilliseptica* genomic DNA exhibited the ability to cause the formation of degradation halos after 3 days of incubation on CGP-overlay agar plates. After subcloning of smaller DNA-fragments in pBluescript SK+, one transformant of *E. coli* XL1-Blue was identified which was also able to form degradation halos on CGP plates. This clone harboured a 2.6-kbp fragment of *P. anguilliseptica* DNA. By DNA sequence analysis of the cloned fragment, the N-terminus of CphE, which was determined by N-terminal amino acid sequence analysis (compare Fig. 9), was rediscovered in antilinear orientation towards the *lacZ* promotor of the vector; the gene can therefore be assumed to be under the control of its own promotor. Upstream of the N-terminus of the mature enzyme, which was determined by N-terminal sequencing, a probable leader peptide of 21 amino acids was found in the deduced amino acid sequence of the gene (see Fig. 9 and Fig. 10). 9-14 base pairs upstream of the methionine codon (ATG) of the leader peptide sequence a purine rich sequence GGAGAA was detected, indicating a potential ribosome binding site (Shine-Dalgarno-sequence) in the complementary mRNA transcript of the gene. An open reading frame of 1,254 bp with TAA as stop codon was identified corresponding to a theoretical protein mass of 42.4 kDa for the mature CphE protein if the mass of the leader peptide (2.4 kDa) is not considered. This corresponds well with the apparent molecular mass of the enzyme (subunit) that was detected by SDS-PAGE (43 kDa, compare Fig. 1). The pI of the mature enzyme, i.e. the extracellular form of CphE, was calculated to be 5.92.

Alignment of the deduced amino acid sequence of *cphE* with proteins exhibiting an acceptable sequence similarity (27-32% in conserved regions) revealed a three amino acid motif most probably representing the catalytical triad also present in other serine type proteases (Fig. 9).

The differences between CphE, CphB enzymes and the most closely related protein, a hypothetical protein of *Caulobacter crescentus* (see Fig. 11), reveal the relatively isolated position for CphE among all known enzymes involved in CGP metabolism.
Employing a newly developed CGP mineral medium, CGP degrading bacteria were isolated from different habitats where cyanobacteria and concomitantly CGP were expected to be present. Every sample applied to the CGP medium, regardless of the samples origin, led to the identification of Gram-negative bacteria capable of utilizing CGP as sole carbon source for growth. This finding revealed the abundance of CGP degrading eubacteria, especially of the genus *Pseudomonas*, to which three isolates (*P. anguilliseptica* BI, *P. alcaligenes* DIII and DIV) were taxonomically assigned in this study. In addition, strain PAS1, which most probably belongs to the genus *Streptomyces*, exhibited good growth on CGP, indicating that the capability to degrade CGP occurs also among Gram-positive bacteria. Occurrence of so many CGP degrading bacteria is not surprising because cyanobacteria represent a large and metabolically highly diverse group of bacteria. Representatives of cyanobacteria occur in almost any aquatic and terrestrial environment exposed to light, and in some of these environments cyanobacteria are the predominant microorganisms. Furthermore, most cyanobacteria are able to synthesize CGP. Moreover, the finding that CGP-like polymers are also synthesized by eubacteria not belonging to the group of cyanobacteria (35), suggests that such degradation mechanisms are even present in obligate heterotrophic microbial communities. In conclusion, CGP is probably an abundant biopolymer in natural environments. However, studies on extracellular degradation of CGP were limited in the past, due to the difficulties to produce sufficient amounts of this biopolymer by cultivation of cyanobacteria.

Because this enzyme, after the cyanophycin synthetase (CphA) and the intracellular CGPase (CphB), is the third bacterial enzyme that is involved in CGP metabolism, and because it is localized extracellularly, the cyanophycinase of *P. anguilliseptica* enzyme was referred to as CphE. As demonstrated in this study, CphE of *P. anguilliseptica* BI was only synthesized if CGP was available as substrate for growth, indicating a specific induction of the enzyme by CGP or its degradation products. Moreover (see Table 1), it also became obvious that there was no correlation between the abilities to degrade CGP and to hydrolyse other polyamide substrates. Most of the other polyamide substrates were only poorly utilized by the employed strains. For example, the natural occurring poly(γ-D-glutamic acid), which is an extracellular polymer of various Gram-positive bacteria (36,37,38,39), was only accepted by isolate DXIII and by *P. alcaligenes* strain BE2, which was previously isolated on poly(γ-D-glutamic acid) containing medium (33). On the other side, by cultivation of non CGP-degrading reference strains including typical protease producing bacteria (i.e. *B. subtilis* and *B. megaterium*) on CGP-overlay agar plates, the resistance of the polymer against typical bacterial proteases, of
which *B. subtilis* produces a great variety (40,41,42,43,44,45), became clearly evident. This is consistent with the finding of the total resistance of CGP to a variety of commercially available proteases (4,9).

Studies on the substrate specificity of CphE of *P. anguilliseptica* BI showed that the enzyme is highly specific for CGP. Therefore, consistent with the findings mentioned above, CGP seems to be exclusively hydrolyzed by employment of specialized CGPases. The lack of release of significant amounts of ninhydrin positive degradation products from bovine casein, BSA or synthetic poly(α,β-D/L-aspartic acid), which is used as a biodegradable substitute for non-degradable polyacrylates in detergents (46), during incubation in the presence of purified enzyme (Fig. 4), clearly demonstrated the high specificity of CphE for CGP. A similar high specificity towards CGP was also described for the intracellular enzyme (CphB) of *Synechocystis* sp. PCC6803 (8).

Purification of CphE from culture supernatants to electrophoretic homogeneity became very efficient by utilizing the high affinity of this CGPase towards immobilized arginine residues, thus allowing purification of CphE by application of only two chromatography steps and with high activity yield. The strong binding of the extracellular CGPase to immobilized arginine residues was not only observed during protein purification employing an arginine-agarose column. Also, binding of CphE to the natural substrate CGP during cultivation occurred with such high affinity that soluble enzyme activity was detected in culture supernatants of CGP degrading bacteria in liquid medium only after all CGP particles in the medium had visibly disappeared, and the enzyme was thereby released from the substrate. After cloning of CphE gene from *P. anguilliseptica* BI and its heterologous functional expression in *E. coli*, sufficient amounts of the enzyme were obtained for further biochemical characterization. HPLC and electrospray ionization mass spectrometry (ESI) analysis identified dipeptides as degradation products of the enzyme reaction; in the case of cyanobacterial CGP β-asp-arg dipeptides occurred (see Fig. 6 and Fig. 7) as described for the intracellular cleavage of CGP in the cyanobacterium *Synechocystis* sp. PCC6803 (8). Degradation experiments employing enzymatically carboxy-terminal L-[U-14C]-arginyl-labelled CGP revealed a continuous and immediate release of radioactive degradation product after addition of CGPase (see Fig. 8). Because β-asp-arg dipeptides were detectable immediately after addition of the enzyme, an exo-degradation mechanism proceeding from the carboxyl-terminus of the CGP molecule and release of the dipeptides by successive cleavage of the α-amide bonds of the polymer backbone, seems to be most likely for CphE.

Molecular characterization of *cphE* revealed a DNA sequence that encodes a protein with a similarity of only 27-28 % to intracellular CGPases (CphB) from cyanobacteria in conserved regions. In contrast to the
intracellular CGPase from *Synechocystis* sp. PCC6803, which has an apparent molecular mass of 27 kDa (8), the molecular mass of extracellular CphE from *P. anguilliseptica* BI was significantly higher (43 kDa). The dendrogram shown in Fig. 11 demonstrates the isolated position of CphE.

The amino acids Ser<sup>169</sup>, Glu<sup>185</sup> and His<sup>222</sup> of CphE may be the catalytic active residues responsible for the hydrolytic cleavage of the α-amide bonds of the polymer backbone (see Fig. 9). Accordingly, the catalytic mechanism is suggested to be that of a serine type protease. This finding is in good agreement with the detected sensitivity of CphE towards serine protease inhibitors (compare Table 3). In contrast to most serine type proteases, the characteristic aspartic acid residue of the catalytic triad is replaced by glutamic acid. The same amino acid replacement was observed for the intracellular CGPase of *Synechocystis* sp. PCC6803 or other cyanobacteria ((8); compare Fig. 5). In the predicted sequence of a hypothetical protein from *Caulobacter crescentus*, which showed the highest similarity to CphE in a NCBI data base search (32% identity over 325 amino acids) and exhibited a similar molecular mass, an aspartic acid residue typical for the catalytic triad of most serine proteases was present (see Fig. 5). Therefore, Asp<sup>188</sup> of CphE, which is according to the alignment close to the position of the proposed catalytic glutamic acid residues of CphB proteins (8), must be considered as another potential residue that is involved in catalysis, instead of Glu<sup>185</sup>. The proposed catalytic aspartic acid residue of the PepE protein, which represents an aspartyl-dipeptidase from *Salmonella typhimurium* (47,48), has been suggested to be in a corresponding position compared to the conserved glutamic acid residues of CphB proteins (8); therefore it seems on the other hand more likely that Glu<sup>185</sup> is catalytically active.

CphE was active when expressed heterologously in *E. coli*, but was not secreted by recombinant cells. The finding of an N-terminal leader peptide in the amino acid sequence deduced from *cphE* (see Fig. 9) suggested an export mechanism for CphE with specific recognition of the signaling peptide and cleavage of the leader sequence during export in *P. anguilliseptica*. Similarities of this leader peptide to that of a potential chemotaxis transducer identified in the *P. aeruginosa* PAO1 genome (49), which is according to its function most probably located in the cytoplasmatic membrane, support the assumption that CphE is also membrane directed. However, the amino acid sequence of the N-terminus of CphE produced in *E. coli* did not deviate from that of native mature (extracellular) CphE, indicating that the suggested leader peptide sequence might be cleaved off in the cytoplasm of *E. coli* cells.

CGPases are most probably commonly employed enzymes for degradation of a widespread and therefore, in cases of biomass degradation, often released biopolymer. This is indicated by its high specificity and
affinity towards CGP-like material. The enzyme makes the dipeptide building blocks quickly available to cells that possess appropriate proteins for the uptake or further cleavage of β-linked amino acid dimers. It should be emphasized that CphE is an extracellular enzyme. It is therefore not involved in the mobilization of intracellular storage polymer CGP; for this, CGP accumulating bacteria possess intracellular CGPases referred to as CphB (8).

REFERENCES
1. Mackerras, A. H., DeChazal, N. M., and Smith G. D. (1990) J. Gen. Microbiol. 136, 2057-2065
2. Liotenberg, S., Campbell, D., Rippka, R., Houmard, J., and deMarsac, N. T. (1996) Microbiology 142, 611-622
3. Allen, M. M., and Weathers, P. (1980) J. Bacteriol. 141, 959-962
4. Simon, R. D., and Weathers, P. (1976) Biochim. Biophys. Acta 420, 165-176
5. Simon, R. D., Lawry, N. H., and McLendon G. L. (1980) Biochim. Biophys. Acta 626, 277-281
6. Ziegler, K., Diener, A., Herpin, C., Richter, R., Deutzmann, R., and Lockau, W. (1998) Eur. J. Biochem. 254, 154-159
7. Oppermann-Sanio, F. B., and Steinbüchel, A. (2002) Naturwissenschaften 89, 11-22
8. Richter, R., Hejazi, M., Kraft, R., Ziegler, K., and Lockau, W. (1999) Eur. J. Biochem. 263, 163-169
9. Simon, R. D. (1987) in The Cyanobacteria (Fay, P., and van Baalen, C., eds) pp. 199-225, Elsevier, Amsterdam
10. Claus, D., and Walker, N. (1964) J. Gen. Microbiol. 36, 107-122
11. Worsey, M. J., and Williams, P. A. (1975) J. Bacteriol. 124, 7-13
12. Frey, K. M., Oppermann-Sanio, F. B., Schmidt, H., and Steinbüchel, A. Appl. Environ. Microbiol.: accepted for publication
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Widdel, F., and Pfennig, N. (1981) Arch. Microbiol. 129, 395-400
15. He, M., Wilde, A., and Kaderbhai, MA (1990) Nucl. Acids. Res. 18, 1660
16. Rao, R. N., Richardson, M. A., and Kuhstoss, S. (1987) Methods Enzymol. 153, 166-198
17. Hohn, B., and Collins, J. (1980) Gene 11, 291-298
18. Simon, R., Priefer, U., and Pühler, A. (1983) Bio-Technol. 1, 784–790
19. Strauss, E. C., Kobori, J. A., Siu, G., and Hood, L. E. (1986) Anal. Biochem. 154, 353–360
20. Huang, X. (1992) Genomics 14, 18-25
21. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997) Nucleic Acids Res. 25, 4876-4882
22. Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M., and Stackebrandt, E. (1996) Int. J. Syst. Bacteriol. 46, 1088-1092
23. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) J. Gen. Microbiol. 111, 1-61.
24. Hai, T., Ahlers, H., Gorenflo, V., and Steinbüchel, A. (2000) Appl. Microbiol. Biotechnol. 53, 383-389
25. Simon, R. D. (1971) Proc. Natl. Acad. Sci. USA 68, 265-267
26. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4417
27. Aboulmagd, E., Oppermann-Sanio, F. B., and Steinbüchel, A. (2000) Arch. Microbiol. 174, 297-306
28. Cole, R. B. (1997) In Electrospray Ionization Mass Spectrometry, John Wiley & Sons, NY
29. Laemmli, U. K. (1970) Nature 227, 680-685
30. Salamone, P. R., and Wodzinski, R. J. (1997) Appl. Microbiol. Biotechnol. 48, 317-324
31. Bradford., M. M. (1976) Anal. Biochem. 72, 248-254
32. Berg, H., Ziegler, K., Piotukh, K., Baier, K., Lockau, W., and Volkmer-Engert, R. (2000) Eur. J. Biochem. 267, 5561-5570
33. Oppermann, F. B, Pickartz, S., and Steinbüchel A., (1998) Polym. Degrad. Stabil. 59, 337-344
34. Jay, F. Y., Coultas, C., and Jones, D. S. (1976) Nucleic Acids Res. 3, 177-190
35. Krehenbrink, M., Oppermann-Sanio, F. B., and Steinbüchel, A. (2001) Arch. Microbiol. 177, 371-380
36. Bovarnick, M.(1942) J. Biol. Chem. 145, 415-424
37. Hanby, W. E., and Rydon, H. N. (1946) Biochem. J. 40, 297-306
38. Hara, T., and Ueda, S. (1982) Agric. Biol. Chem. 46, 2275-2281
39. Birrer, G. A., Cromwick, A. M., and Gross, R. A. (1994) Int. J. Biol. Macromol. 16, 265-275
40. Stahl, M. S., and Ferrari, E. (1984) J. Bacteriol. 158, 411-418
41. Yang, M., Farrari, E., and Henner (1984) J. Bacteriol. 160, 15-21
42. Sloma, A., Rufo, G. A., Rudolph, C. R., Sullivan, B. J., Theriault, K. A., and Pero, J. (1990) J. Bacteriol. 172, 1470-1477
43. Rufo, G. A., Sullivan, B. J., Sloma, A., and Pero, J. (1990) J. Bacteriol. 172, 1019-1023
44. Sloma, A., Ally, A., and Pero, J. (1988) J. Bacteriol. **170**, 5557-5563

45. Tran, L., Wu, X. C., and Wong, S. L. (1991) J. Bacteriol. **173**, 6364-6372

46. Alford, D. D., Wheeler, A. P., and Pettigrew, A. (1994) J Environ. Polym. Degrad. **2**, 225-236

47. Carter, T. H., and Miller, C. G. (1984) J. Bacteriol. **159**, 453-459

48. Miller, C. G. (1998) Dipeptidase E. *Handbook of Proteolytic Enzymes* pp. 1557-1558. Academic Press, San Diego

49. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R. M., Smith, K. A., Spencer, D. H., Wong, G. K. S., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E. W., Lory, S., and Olson, M. V. (2000) Nature **406**, 959-964

50. Tanguy-Rougeau, C., Wesolowski-Louvel, M., and Fukahara, H. (1988) FEBS Lett. **234**, 464-470

51. Latchinian-Sadek, L., and Thomas, D. Y. (1993) J. Biol. Chem. **268**, 534-540

**Footnotes**

The nucleotide sequence(s) reported in this paper have been submitted to the NCBI database with accession numbers AF439803 and AY065671.

The abbreviations used are: CGP, cyanophycin granule polypeptide (cyanophycin); PMSF, phenylmethylsulfonylfluoride; bp, base pair(s); EDTA, ethylenediamine-tetraacetic acid; ESI, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis, PGA, poly(γ-D-glutamic acid); KU, Klett units, OD, optical density; OPA, o-pthalialdehyde

**Acknowledgments**—We thank Dr. Tran Hai for the provision of the strains PAS I and *Synechocystis* PCC6308 (SK19-1) and Francis Hezayen for the provision of poly(γ-D-glutamic acid). We gratefully acknowledge Martin Krehenbrink for the performance of the CGP-labelling experiments. Generous support by Bayer (Leverkusen) is gratefully acknowledged.
Legends to Figures

Fig. 1: Detection of the extracellular cyanophycinase of *Pseudomonas anguilliseptica* strain BI. A) Halo formation during isolation of bacteria on CGP-mineral medium. B) Routine test for enzyme activity in chromatography fractions. C) Nonspecific protein staining (Coomassie; (26)) of samples from FPLC-purification after separation in SDS-polyacrylamide gels; lane 1: molecular mass standard proteins, lane 2: supernatant of CGP-culture broth, lane 3: protein pattern after anion exchange chromatography and lane 4: after arginine-agarose affinity chromatography; 10 µg protein of each sample was applied to the gel D) Activity staining (black arrow) after SDS-PAGE, in-gel renaturation and subsequent application of a CGP-agar layer (lane1), lane 2: molecular mass standard proteins.

Fig 2. Taxonomic classification of the isolated *P. anguilliseptica* strain BI. The affiliation of strain BI is based on 16S rRNA gene sequence homologies to closely related bacterial strains, i.e. strains of the genus *Pseudomonas*. The new isolate BI groups with the three *P. anguilliseptica* strains from the NCBI database (98% sequence identity), including the type strain NCIMB 1949. Many other strains of the genus *Pseudomonas*, of which some examples are depicted above, showed sequence identities of 95-96% to strain BI. The bar represents the number of expected changes per sequence position of those positions changing at the median rate.

Fig. 3. Growth of *Pseudomonas anguilliseptica* BI in liquid medium B. Cells of strain BI were grown in 30 ml cultures in 300-ml Klett flasks on either 0.5% (w/v) cyanophycin (*M_* = 25-100 kDa; ■), arginine (O), aspartic acid (□), arginine + aspartic acid (Δ) or citrate (♦). In case of using arginine and aspartic acid as cosubstrates (together 0.5%, w/v), these amino acids were added according to their proportional masses in the cyanophycin molecule (i.e. 57% (w/w) arg : 43% (w/w) asp). The sterile control (▲) contained 0.5% (w/v) of citrate. The cultures were inoculated from a 10 ml over night citrate preculture using 1 ml as inoculum. The incubation of the cultures was done at 30°C on a rotary shaker (120 rpm). Growth was recorded in a Klett-Summerson photometer.
Fig. 4. **Substrate specificity of the CGPase (CphE).** Recombinant CphE (1.6 µg purified enzyme) was incubated for 120 minutes with 1 mg of the indicated polypeptides. The formation of hydrolysis products was detected by employing the ninhydrin method and measuring the increase of ninhydrin activity at 570 nm; poly(Asp) = poly(α,β-D/L-aspartic acid), BSA = bovine serum albumin, CGP = cyanophycin.

Fig. 5. **Decrease of the molecular weight of CGP during incubation of the polymer in the presence of purified recombinant CphE.** CGPase was inactivated before electrophoresis (SDS-PAGE) by incubation at 70°C for 5 minutes. Lanes 1 + 11 = molecular weight standard proteins, lane 2 = 25 µg cyanobacterial CGP in 50 mM sodium phosphate buffer (pH 8.3); lanes 3 – 10: 5 min, 11 min, 17 min, 25 min, 37 min, 51 min, 69 min and 165 min after the addition of 16 µg purified recombinant CphE. Cyanophycin was visualized by Coomassie staining (26).

Fig. 6. **Detection of degradation products of the CGPase reaction by HPLC-analysis.** Analysis of products of the degradation of (A) cyanobacterial CGP isolated from *Synechocystis* PCC6308 and (B) of recombinant CGP isolated from *E. coli* DH1 (pMa/c5-914::cphA) containing lysine residues randomly incorporated as arginine substitutes.

Fig. 7. **Positive ion ESI/MS analysis of the CGP degradation product (A) and ESI/MS/MS analysis (B) of the m/z 290 peak from (A).** (A) The ESI/MS peak m/z 290 corresponds to the mass of the protonated β-asp-arg dipeptide [M + H]⁺. (B) The fragmentation pattern of the [M + H]⁺ ion from (A) is in accordance with the structure of the β-asp-arg dipeptide. The loss of 115 and 174 atomic mass units indicates fragmentation at the β-amide bond. m/z 273 and m/z 158 correspond to a loss of an ammonia moiety from the [M+H]⁺ and the [M+H-115]⁺ respectively.

Fig 8. **Degradation of L-[U-14C]-arginy-l-labelled CGP.** The release of labelled hydrolysis products is shown as an increase in scintillation counts.
Fig. 9. Alignment of deduced amino acid sequence of CGPase CphE from *P. anguilliseptica* strain BI with protein sequences of highest similarity (NCBI database). A potential signaling peptide preceeding the N-terminus of CphE is underlined. The N-terminus (shaded in light grey) was determined by peptide sequencing of purified native CGPase. The proposed residues of the catalytic triad are shaded in grey and are indicated by the symbols ▼, ∀ and ▽ for serine, aspartic acid (or glutamic acid) and histidine. A potential alternative catalytic aspartic acid residue of CphE is shaded in light grey. The conserved Gly-Xaa-Ser-Xaa-Gly motif of serine type proteases is boxed. Three tryptophan residues whose oxidation could have lead to enzyme inactivation (compare Table 3) are underlined and in bold letters. For sequence alignment the ClustAlX 1.8 program was employed (21). PCC6308 = CphB from *Synechocystis* sp. PCC6308; PCC6803 = CphB from *Synechocystis* sp. PCC6803; *A. variabilis* = CphB from *Anabaena variabilis*; *C. crescentus* = hypothetical protein from *Caulobacter crescentus*; PepE = alpha-aspartyl dipeptidase from *Salmonella typhimurium* LT2.

Fig. 10. Similarity of the N-terminal leader peptide of CphE (deduced amino acid sequence) from *P. anguilliseptica* strain BI to N-terminal amino acid sequences of other proteins. Amino acids present in two or in all sequences depicted are shaded in grey. At the amino acid residue positions -1 and -3 short chained amino acids are present (potential recognition site for peptide cleavage). A hydrophilic arginine residue and serine residues preceeding a hydrophobic leucine rich domain was found in CphE and in the potential chemotaxis transducer of *Pseudomonas aeruginosa* PAO1(49). In the sequence of the Kex1 protein (precursor of a subtilisin type serine protease from *Kluveromycetes lactis* (50)) two serine residues followed by a leucine and isoleucine rich hydrophobic domain were detected as well (compare boxed hydrophobic sequences).
Amino acid sequence similarity of CphE to intracellular CGPases (CphB) and other proteins.

The cyanobacterial CGPases form a phylogenetically related group. Strongest similarity of CphE was found to a hypothetical protein of *Caulobacter crescentus* (32% identity over 325 amino acids, NCBI database search). The isolated position of CphE among CGPases is revealed by the distance between CphE and any other “related” protein, including the alpha-asparyl dipeptidase (pepE) from *Salmonella typhimurium* previously described as a related protein of intracellular CGPases (8).
Table 1: Growth of CGP-degrading bacteria on compounds related to cyanophycin or other polyamide substrates. Strain AV$_N$ was isolated from Baltic sea water; strains BI and BII were obtained from pond sediments and the strains of the “D-series” were isolated from sewage sludge. *Bacillus subtilis* 168 was used as a control.

| Isolate | Strain | Growth on carbon source$^b$ |
|---------|--------|-----------------------------|
|         |        | BSA | PGA | Asp | Arg | Asp + Arg | Citrate |
| AV$_N$  | n.d.$^a$ | -   | -   | ++  | ++  | ++        | +++     |
| BI      | *Pseudomonas anguilliseptica* | -   | -   | -   | ++  | ++        | +++     |
| BII     | n.d.$^a$ | -   | -   | ++  | ++  | ++        | +++     |
| DIII    | *Pseudomonas alcaligenes* | -   | -   | +++ | +++ | +++       | +++     |
| DIV     | *Pseudomonas alcaligenes* | -   | -   | +++ | +++ | +++       | +++     |
| DX      | n.d.$^a$ | -   | -   | +   | ++  | ++        | +++     |
| Dxi     | n.d.$^a$ | -   | -   | +   | ++  | ++        | +++     |
| DXI$_i$ | n.d.$^a$ | -   | -   | +   | ++  | +++       | +++     |
| DXIII   | n.d.$^a$ | -   | +++ | +++ | ++  | +++       | +++     |
| BE2$^*$ | *Pseudomonas alcaligenes* | +   | +   | +++ | +++ | +++       | +++     |
| PAS I   | n.d.$^a$ | +++ | -   | -   | ++  | +++       | ++      |
| -       | *Bacillus subtilis* | +   | -   | -   | ++  | +++       | +++     |

$^a$ = taxonomically not determined. Growth was qualitatively estimated as follows: -, no growth; +, poor growth; ++, moderate growth; ++++, good growth. Strains were grown on basic inorganic medium B with concentrations of 0.25% of each of the applied carbon sources.$^*$ Strain BE2 was isolated in a screening for poly(γ-D-glutamic acid) degrading bacteria (33); BSA = bovine serum albumin, PGA = poly(γ-D-glutamic acid), Asp = aspartic acid, Arg = arginine.
Table 2: Substrate utilization of CGP-degrading bacteria in the API 20NE physiological determination assay. Strain AVN was isolated from Baltic sea water; strain BI was obtained from pond sediments and the strains of the “D-series” were isolated from sewage sludge. The following tests were negative for all four isolates and are not shown in the table: denitrification test, indole formation (tryptophan conversion); urease test, β-glucosidase test, β-galactosidase test, anaerobic utilization of glucose (fermentation), aerobic substrate utilization tests: glucose, arabinose, mannose, manitol, N-acetyl-glucosamine, maltose, gluconate, adipate, phenyl-acetate

| Isolate | Strain               | NO₂ | ADH | GEL | OX | MLT | CIT | CAP |
|---------|----------------------|-----|-----|-----|----|-----|-----|-----|
| AVN     |                      | -   | -   | -   | +  | +   | +   | +   |
| BI      | Pseudomonas           |     |     |    | +  | +   | +   | +   |
|         | anguilliseptica*     |     |     |    |    |     |     |     |
| DIII    | Pseudomonas           |     |     | +  | +  | +   | +   | +   |
|         | alcaligenes           |     |     |    |    |     |     |     |
| DIV     | Pseudomonas           |     |     | +  | +  | +   | +   | +   |
|         | alcaligenes           |     |     |    |    |     |     |     |

a = taxonomically not determinable with this test. * taxonomically determined by 16S-rDNA sequence analysis; b API 20 NE-Assays: NO₂ = NO₃-reduction $\rightarrow$ NO₂, ADH = arginine dihydrolase, GEL = gelatinase, OX = oxidase assay; aerobic substrate utilization: MLT = malate, CIT = citrate, CAP = caprate
Table 3: Inhibition of the CGPase by group specific protease inhibitors. CGPase from *P. anguilliseptica* BI was incubated in sodium phosphate buffer (pH 8.3) for 30 minutes at room temperature in the presence of the listed inhibitors and subsequently applied onto A) CGP-overlay agar plates or to B) suspended CGP. Inhibition of the enzyme was detected by delayed or total prevention of halo formation on CGP-overlay plates and by HPLC analysis (detection of the degradation product after OPA-derivatization, compare Fig. 5). The control was without inhibitor.

| Inhibitor specificity | Inhibitor (solvent) | Concentration of inhibitor in the assay [mM] (low → high) | A) CGP plate assay | B) HPLC analysis |
|------------------------|---------------------|----------------------------------------------------------|------------------|------------------|
|                        |                     | halo formation (low inhibitor concentration) | halo formation (high inhibitor concentration) | inhibition [%]§ (low inhibitor concentration) | inhibition [%]§ (high inhibitor concentration) |
| Thiol proteases        | Leupeptin (H₂O)     | 0.001-0.01                                            | +                | +                | 13               | 13               |
| Metalloproteases       | EDTA (H₂O)          | 30-60                                                  | +                | +                | 46*              | 49*              |
| Serine proteases       | Pefabloc® (H₂O)     | 8-80                                                   | +                | -                | 13.6             | 92               |
|                        | PMSF (in isopropanol)| 1-10                                                   | +                | +                | 28               | 63               |
| Tryptophan residues    | N-Bromo-succinimide (H₂O) | 1-5                                                    | -                | -                | 99               | 100              |
| control                |                     |                                                        | -                | +                | 0                |                  |

* values are not due to inhibition; the application of EDTA interfered with the OPA derivatization during HPLC analysis (formation of precipitates)

§ percentage values of inhibition refer to the control, i.e. activity of CGPase in the absence of an inhibitor
Graph showing Ninhydrin activity [570 nm] for different substrates:

- CGP: 2.02
- Bovine casein: 0.08
- BSA: 0.13
- poly(Asp): 0
cphE leader peptide  MIRSFIRSSALLALLPVTCYS
pot. chemotaxis transducer  MMRLTLKSKVLLLAMVPULLFA
Kex1 protease precursor  ----MILSFLMLIALIASYIG
Isolation of cyanophycin degrading bacteria - cloning and characterization of an extracellular cyanophycinase gene (cphE) from Pseudomonas anguilliseptica BI
Martin Obst, Fred Bernd Oppermann-Sanio, Heinrich Luftmann and Alexander Steinbüchel

J. Biol. Chem. published online May 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112267200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts