

Cloning of a Nitrilase Gene from the Cyanobacterium *Synechocystis* sp. Strain PCC6803 and Heterologous Expression and Characterization of the Encoded Protein

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The gene encoding a putative nitrilase was identified in the genome sequence of the photosynthetic cyanobacterium *Synechocystis* sp. strain PCC6803. The gene was amplified by PCR and cloned into an expression vector. The encoded protein was heterologously expressed in the native form and as a His-tagged protein in *Escherichia coli*, and the recombinant strains were shown to convert benzonitrile to benzoate. The active enzyme was purified to homogeneity and shown by gel filtration to consist probably of 10 subunits. The purified nitrilase converted various aromatic and aliphatic nitriles. The highest enzyme activity was observed with fumarodinitrile, but also some rather hydrophobic aromatic (e.g., naphthalenecarbonitrile), heterocyclic (e.g., indole-3-acetonitrile), or long-chain aliphatic (di-)nitriles (e.g., octanoic acid dinitrile) were converted with higher specific activities than benzonitrile. From aliphatic dinitriles with less than six carbon atoms only 1 mol of ammonia was released per mol of dinitrile, and thus presumably the corresponding cyanocarboxylic acids formed. The purified enzyme was active in the presence of a wide range of organic solvents and the turnover rates of dodecanoic acid nitrile and naphthalenecarbonitrile were increased in the presence of water-soluble and water-immiscible organic solvents.

Nitrilases that hydrolyze organic nitriles to carboxylic acids and ammonia are a commercially very interesting group of enzymes because nitriles are important intermediates in the chemical synthesis of various products. Several biotransformation processes have been described which utilize the chemo-, regio-, or enantioselectivity of nitrilases (3, 9, 17, 25, 26, 34). All nitrilases studied in some detail contain a cysteine residue in their catalytic center and catalyze the hydrolysis of their substrates via the intermediate formation of a covalently bound acyl residue (Fig. 1) (15, 30). Nitrilases from a variety of prokaryotic and eukaryotic organisms have been described (10, 11, 33, 39). The main source of nitrilases are bacterial species, which are usually obtained by enrichments from environmental samples with the nitriles as sole source of nitrogen. These enrichments result in most cases in the isolation of bacteria that either belong to the *Proteobacteria* (such as *Acinetobacter*, *Alcaligenes*, or *Pseudomonas* strains) (21, 22, 40, 41) or to the high-GC gram-positive bacteria (mostly rhodococci) (10, 16, 18, 21, 36). Many of the known nitrilases possess considerable disadvantages (e.g., insufficient stability or selectivity or low specific activities) that prevent their industrial application, and there is therefore a constant demand for new nitrilases. Because the traditional enrichment strategies usually result in the isolation of a rather restricted group of microorganisms, we decided to perform an *in silico* screening for new nitrilases from DNA sequences obtained in the course of genome projects and deposited in the databases. Therefore, these da-

tases were screened for the presence of putative nitrilase genes in mesophilic microorganisms that are only distantly related to the typical nitrilase-containing organisms previously described. These sequence comparisons suggested that an interesting nitrilase might be present in the cyanobacterium *Synechocystis* sp. strain PCC6803, which was therefore investigated in the present study.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* JM109 was used for all cloning experiments. The strain was routinely cultured in Luria-Bertani (LB) medium (plus 100 µg of ampicillin/ml if appropriate). The plasmids pJOE2702 and pJOE2775 have been described before (37, 38).

Amplification of the nitrilase gene by PCR and subsequent cloning of the gene. The gene was amplified from the total DNA of *Synechocystis* sp. strain PCC6803 (kindly provided by Anke Engels, University of Tübingen, Tübingen, Germany) by using the two oligonucleotide primers 5'-TTG GAT CCT TAA TGG CTA AAA ATC CG-3' (SycnitBI) and 5'-AAA AAT TCA TAT GCT GAA TTA TAC AAA AAA T-3' (SycNitNu), which incorporated *Bam*HI and *Nde*I restriction sites, respectively. The amplification was performed in a final volume of 40 µl, and the reaction mixtures contained template DNA, 10 pmol of each primer (MWG Biotech, Ebersberg, Germany), a 220 µM concentration of each deoxynucleotide triphosphate, 1× PCR buffer, 2 U of *Taq* DNA polymerase (Boehringer Roche), and 5 mM MgCl₂.

The following PCR program was used for the amplification of an 1,041-bp DNA fragment: 3 min of denaturing at 93°C; 30 cycles of denaturing (1 min at 93°C), annealing (1 min at 40°C), and polymerization (25 s at 72°C) followed. The program was completed by an additional polymerization step (5 min at 72°C).

The amplified 1,041-bp DNA fragment was cut with *Nde*I and *Bam*HI, and the resulting fragment was ligated into vector pJOE2702, which had been cut with the same restriction enzymes. The resulting plasmid was designated pDHE21.

In order to obtain a nitrilase derivative with a His₆ tag, the nitrilase gene was amplified with the primer pair 5'-TTG GAT CCA TGG CTA AAA ATC GGA TT-3' (SycnitBIHis) and SycNitNu (see above) by using pDHE21 as a template under the conditions described above. The amplified fragment was also cut with

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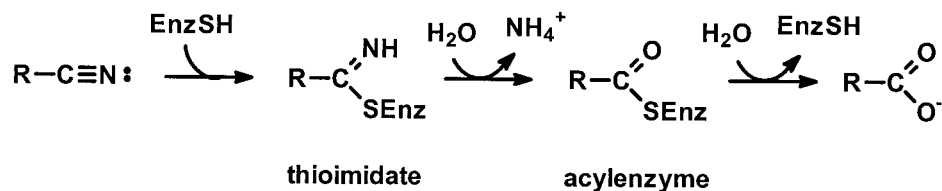


FIG. 1. Proposed mechanism for the nitrilase reaction (30).

NdeI and *BamHI*, and the resulting fragment was ligated into the *NdeI/BamHI*-cut vector pJOE2775, giving pDHE22.

Expression of the nitrilase in *E. coli*. For the induction of the nitrilase in the recombinant *E. coli* strains, precultures (2 ml each) were grown in LB-ampicillin medium. From these precultures, Erlenmeyer flasks (usually with a volume of 300 ml) containing 0.2% (wt/vol) L-rhamnose in LB-ampicillin medium were inoculated to an initial optical density at 546 nm of 0.02. The bacterial cultures were incubated at 30°C on an orbital shaker at 120 rpm. The cells were harvested ca. 13 h later.

For the preparation of resting cells, exponentially growing bacterial cultures expressing the nitrilase activity were harvested by centrifugation (15 min, 4°C, 9,500 × g) and washed and resuspended in 50 mM Na-K phosphate buffer (pH 7.0).

Analytical methods. Benzonitrile and benzoate were analyzed by reversed-phase high-pressure liquid chromatography (HPLC) by using Millennium Chromatography Manager 2.0 equipped with a photodiode array detector model 996 and HPLC pump model 510 (Waters Associates, Milford, Mass.). A reversed-phase column (125 by 4.0 mm [internal diameter]; Grom, Herrenberg, Germany) packed with 5- μm particles of Grom-Sil 100 Octyl was used. The separated compounds were detected photometrically at 210 nm with a photodiode array detector. The eluting solvent system was composed of 79.7% (vol/vol) water, 20% (vol/vol) acetonitrile, and 0.3% H_3PO_4 . The usual flow rate was 0.7 ml/min.

Enzyme assays. The standard assays contained 50 mM Na-K phosphate buffer (pH 7.0), benzonitrile (10 mM), and preparations of the purified nitrilase or resting cells of the recombinant *E. coli* strains. For the preparation of resting cells, exponentially growing bacterial cultures expressing the nitrilase activity were harvested by centrifugation (15 min, 4°C, 9,000 × g) and then washed and resuspended in 50 mM Na-K phosphate buffer (pH 7.0). The reaction mixtures were incubated in a Thermomixer (CLF-Schuttron, Emersacker, Germany) at 30°C and 300 rpm. Aliquots (usually 50 to 100 μl) were taken after different time intervals, the reactions were stopped by the addition of 10% (vol/vol) 1 M HCl, and the cells or precipitated proteins were removed by centrifugation (16,000 × g, 2 min). The turnover of benzonitrile was usually analyzed by HPLC (see above). The conversion of other (especially aliphatic nitriles) was usually determined by the quantitation of the amount of ammonia (in 1 ml of reaction volume), which was enzymatically released from the substrates by using a commercially available ammonia test system relying on the indophenol method (Spectroquant 14752; Merck, Darmstadt, Germany). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of product (benzoate or ammonia) per min.

Preparation of cell extracts. The cultures of *E. coli* JM109(pDHE21) or *E. coli* JM109(pDHE22) were harvested by centrifugation (9,500 × g, 15 min, 4°C), washed, and resuspended in Na-K phosphate buffer (50 mM, pH 7.5) or Tris-HCl (50 mM, pH 7.5), respectively. The cells were disrupted by using a French press (Aminco, Silver Springs, Md.) at 80 MPa. Cell debris was removed by centrifugation at 110,000 × g for 60 min at 4°C. Protein was determined by the method of Bradford (2) with bovine serum albumin as a standard.

Purification of the native enzyme. Protein was purified at 4°C by use of a fast-performance liquid chromatography system consisting of a LCC 501 plus controller, two P-500 pumps, a UV-1 monitor, a REC-112 recorder, and a FRAC-100 autosampler from Pharmacia (Uppsala, Sweden).

Crude extract was applied to a Q Sepharose column (XK 16/10; Pharmacia). The adsorbed proteins were eluted with a linear gradient of 0 to 400 mM NaCl in Tris-HCl (50 mM, pH 7.5) at a flow rate of 2 ml/min. Fractions (1.5 ml) were collected, and enzyme activity was determined with benzonitrile as the substrate by using the Spectroquant system. The nitrilase was eluted at a concentration of ca. 0.2 M NaCl. The active fractions were pooled, transferred to a hydroxyapatite column (XK 16/10, ceramic hydroxyapatite type I, 20- μm particle size; Bio-Rad), and eluted with a linear gradient (10 to 400 mM) of K phosphate buffer (pH 7.5) at a flow rate of 1 ml/min, and fractions of 0.9 ml were then collected. The

enzyme activity eluted at a concentration of 135 mM phosphate. The pooled fractions (1.8 ml) were concentrated by ultrafiltration (Vivaspin 2; Vivascience, Hannover, Germany) to a volume of 0.6 ml, and proteins were finally chromatographed on a Superdex 200 Prep-Grade HiLoad column (HR 16/60; Amersham Biosciences) by using Na-K phosphate buffer (50 mM, pH 7.3) plus 150 mM NaCl as the elution buffer.

Purification of the His-tagged enzyme. Cell extracts of *E. coli* JM109 (pDHE22) were prepared in Tris-HCl buffer (50 mM, pH 7.5) as described above. The nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) was suspended in Tris-HCl (20 mM, pH 7.5) and transferred (4 ml) to an empty 10 ml-polypropylene column. The filled column was equilibrated with a buffer system (pH 7.5) consisting of Tris-HCl (50 mM), NaCl (300 mM), and imidazole (40 mM). The cell extracts (50 to 70 mg of protein) were applied to the column, and the protein was eluted with subsequent steps of the Tris-HCl-NaCl buffer (2 to 4 ml each) with increasing imidazole concentrations (100, 150, 300, and 500 mM). The fractions with nitrilase activity eluted at an imidazole concentration of 500 mM. This fraction was finally concentrated by ultracentrifugation (Vivaspin2, 10,000 MWCO PES; Vivascience) and resuspended in Na-K phosphate buffer (50 mM, pH 7.4) plus NaCl (300 mM) in order to remove the imidazole which disturbed the ammonia test.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20). Gels were silver stained by the method of Shevchenko et al. (35).

Determination of molecular weight. The relative molecular mass of the native enzyme was determined by gel filtration by using a Superdex 200 Prep-Grade HiLoad 16/60 column (Pharmacia). For the determination of the molecular mass of the nitrilase, the column was calibrated with thyroglobulin (M_r 670,000), ferritin (M_r 440,000), catalase (M_r 232,000), aldolase (M_r 158,000), and albumin (M_r 67,000) as references (from the high- and low-molecular-weight gel filtration kits from Amersham Pharmacia).

Substrate specificity of the purified nitrilase. The purified enzyme (20 μg) was incubated for 30 to 240 min in 0.2 ml of Na-K phosphate buffer (pH 7.0, 50 mM) plus 5% (vol/vol) methanol and 5 mM concentrations of the respective nitriles. The reaction was stopped by the addition of 20 μl of 1 M HCl, and precipitated protein was removed by centrifugation in an Eppendorf centrifuge (16,000 × g, 2 min). The supernatant was diluted with H_2O to 1 ml, and the ammonia content was determined as suggested by the manufacturer of the test kit (Spectroquant 14752; Merck). A control experiment without added enzyme was performed for each substrate, and the amount of ammonia found in the reaction mixture was corrected for the value found in the control experiment.

Determination of the temperature and pH optima. For the determination of the temperature maximum, the purified nitrilase (80 μg of protein) was incubated in 0.34 ml of Na-K phosphate buffer (50 mM, pH 7.0) with benzonitrile (10 mM) in Eppendorf reaction tubes in a thermoshaker at 35, 40, 45, 50, or 55°C. Aliquots (50 μl) were taken after 10, 20, 40, 60, and 120 min, and the formation of benzoate was quantified by HPLC.

The pH optimum of the nitrilase was determined by using a universal buffer according to the method of Theorell and Stenhagen (32). The buffer consisted of a mixture of citric acid, phosphoric acid, boric acid, and NaOH, which was titrated with HCl to obtain the desired pH value (32). The purified nitrilase (55 μg of protein) was incubated in 0.1 ml of the respective buffer with benzonitrile (10 mM) in Eppendorf reaction tubes in a thermoshaker at 30°C. The reactions were stopped after 60 min by the addition of 10 μl of 1 M HCl, and the formation of benzoate was quantified by HPLC.

Chemicals. The nitriles were obtained from Fluka (Neu-Ulm, Germany), Lancaster (Mühlheim-Main, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Bayer (Leverkusen, Germany), Sigma-Aldrich (Munich, Germany), and the Sigma-Aldrich Library of Rare Chemicals (Milwaukee, Wis.).

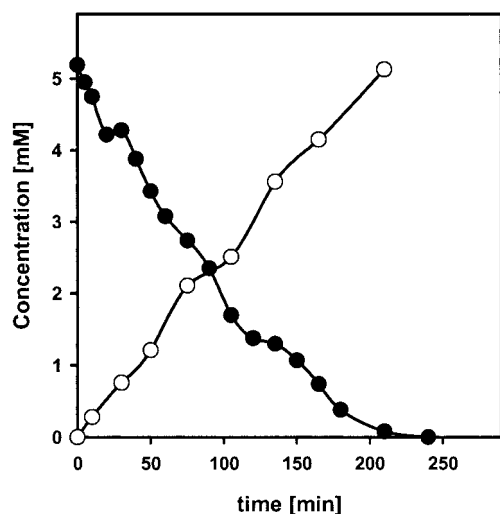


FIG. 2. Demonstration of a nitrilase activity in *E. coli*(pDHE22) which harbors the recombinant nitrilase from *Synechocystis* sp. strain PCC6803. The reaction mixture contained, in a total volume of 5 ml, 50 mM Na-K phosphate buffer, 5 mM benzonitrile, and resting cells of *E. coli*(pDHE22) corresponding to an optical density at 546 nm of 4. Aliquots (50 μ l each) were removed after different time intervals, the reaction was terminated by the addition of 5 μ l of 1 M HCl, and cells and precipitated protein were removed by centrifugation (2 min, 16,600 \times g). The conversion of benzonitrile to benzoate was determined by HPLC.

RESULTS

Cloning of the nitrilase gene from *Synechocystis* sp. strain PCC6803. The published genome sequence of the cyanobacterium *Synechocystis* sp. strain PCC6803 (NCBI accession number D64005) contained an open reading frame encoding a putative nitrilase. The deduced protein sequence (NCBI accession no. NP_442646) demonstrated ca. 36% sequence identity to the nitrilase from *Rhodococcus rhodochrous* J1 (NCBI accession no. A45070). The putative nitrilase gene from *Synechocystis* sp. strain PCC6803 contained at the 5' end a set of two ATG triplets separated by 15 nucleotides. It appeared more probable that the second ATG triplet functioned in vivo as a start codon because a putative Shine-Dalgarno sequence was found upstream and also the sequence alignment of the corresponding gene product was in better agreement with published nitrilase sequences. The corresponding gene was therefore amplified from the complete DNA of strain PCC6803 by PCR as described in Materials and Methods and expressed under the control of a rhamnose-inducible promoter (in plasmid pDHE21) or under the same promoter as a (carboxy-terminally) His-tagged protein (in plasmid pDHE22).

Demonstration of nitrilase activity. The putative nitrilase was induced in *E. coli* JM109(pDHE21) by the addition of rhamnose (see Materials and Methods), and resting cells were then incubated with benzonitrile. The recombinant cells converted benzonitrile almost stoichiometrically to benzoate with a specific activity corresponding to 0.02 U/mg of protein (Fig. 2). In a control experiment with the plasmid-free strain *E. coli* JM109, no turnover of benzonitrile was detected.

Purification of the nitrilase. The nitrilase was purified from cell extracts of the recombinant *E. coli* strains carrying plas-

TABLE 1. Purification of nitrilase from *Synechocystis* sp. strain PCC6803^a

Purification step	Total protein (mg)	Sp act (U/mg)	Total activity (U)	Recovery (%)	Purification (fold)
Crude extract	227	0.03	6.8	100	1.00
Ion exchange	40	0.11	4.4	65	3.7
Hydroxyapatite	10.7	0.13	1.4	21	4.3
Gel filtration	1.7	0.21	0.36	5.3	7.0

^a Experimental details are given in Materials and Methods. The enzymatic activity was determined with benzonitrile as a substrate, and the amount of ammonia released was spectrophotometrically determined.

mids pDHE21 (wild-type nitrilase) or pDHE22 (carrying the carboxy-terminal His tag) as described in Materials and Methods. The purification of the wild-type enzyme by anion-exchange chromatography, hydroxyapatite chromatography, and gel filtration resulted in a pure enzyme preparation (as shown by SDS-PAGE and silver staining) with a rather low yield and low specific activity (0.21 U/mg of protein) for the conversion of benzonitrile (Table 1). The purified nitrilase (7 μ g of protein) was incubated in Na-K phosphate buffer (50 mM, pH 7.0) with benzonitrile (10 mM) at 30°C. The turnover of the substrate was analyzed for several hours, and it was observed that the increase in the formation of benzoate was almost linear for at least 5 h before the reaction slightly slowed down.

The purification of the His-tagged protein variant from *E. coli* JM109(pDHE22) by nickel-agarose chromatography resulted in a nitrilase preparation with an \sim 2 times-higher specific activity. This might suggest that the nitrilase was partially inactivated during the time-consuming multistep purification of the native enzyme.

Enzyme stability. The His-tagged enzyme was moderately stable during storage under different conditions. After 10 days of storage at 5°C in Na-K phosphate buffer (50 mM, pH 7.5) or in the same buffer plus 1 mg of bovine serum albumin/ml, 0.3 M NaCl, or 1% (vol/vol) PEG 4000, ca. 20, 40, 15, or 10%, respectively, of the initial activity could be detected. After storage at -20°C under the same conditions, between 58 and 68% of the initial activity was recovered.

Molecular weight and subunit structure. The purified wild-type enzyme gave a single band by SDS-PAGE (2 to 6 μ g of enzyme) with a molecular weight of ca. 40,000. A protein with the same molecular weight was already very prominent in the cell extract. The molecular weight of the holoenzyme was estimated by gel filtration to be 390,000. Therefore, it can be assumed that the enzyme consists of ca. 10 subunits of identical size.

Temperature and pH optimum. The temperature optimum of the nitrilase was at 50°C if the reaction was monitored only for 10 min, but the enzyme rapidly lost its activity under these conditions. If the reaction was monitored for 2 h, a temperature optimum of 40 to 45°C was determined, but also under these conditions only 60% of the initial activity could be recovered at the end of the incubation period. The enzyme showed maximal activity at pH 7.0. At pH 5.0 and 8.0 ca. 50 to 65% of the maximal activity was found. No enzymatic activity was detected at pH 3 or 12.

TABLE 2. Conversion of various nitriles by purified His-tagged nitrilase from *Synechocystis* sp. strain PCC6803

General compound group	Substrate	Relative activity (%) ^a
Benzene derivatives	Benzonitrile	100
	4-Aminobenzonitrile	28
	Benzylcyanide	52
	2-Chlorobenzylcyanide	6
	3-Chlorobenzylcyanide	316
	4-Chlorobenzylcyanide	11
	2-Methylbenzylcyanide	207
	4-Hydroxybenzylcyanide	10
2-3 Ring systems	2-Naphthalenecarbonitrile	757
	2-Naphthaleneacetonitrile	8
	9-Anthracenecarbonitrile	10
	3-Phenoxyphenylacetonitrile	35
Heterocyclic compounds	2-Thienylacetonitrile	311
	3-Thienylacetonitrile	102
	3-Cyanopyridine	245
	Indole-3-acetonitrile	354
Saturated aliphatic mononitriles	Propionitrile	33
	2-Chloropropionitrile	270
	3-Dimethylaminopropionitrile	8
	Butyronitrile	109
	Valeronitrile	222
	Isovaleronitrile	9
	2-Methylglutaronitrile	394
	Octanoic acid nitrile	303
Dodecanoic acid nitrile	36	
Saturated aliphatic dinitriles	Adipinic acid dinitrile	528
	Octanoic acid dinitrile	588
	Decanoic acid dinitrile	476
Unsaturated aliphatic mononitriles	Acrylonitrile	68
	2-Butenenitrile	49
	3-Butenenitrile	409
Unsaturated aliphatic dinitriles	Fumarodinitrile	12,000
	3-Hexenoic acid dinitrile	14

^a The activity with benzonitrile was 100%. Less than 5% relative activity was found with the following substrates. 2-Ethyl-2-phenylbutyronitrile, 2-phenylvaleronitrile, 2,6-dichlorobenzylcyanide, naproxen nitrile, ketoprofen nitrile, acetonitrile, malononitrile, 2-methyl-3-butenenitrile, diethylidicyanofumarate, isobutyronitrile, 2-methylbutyronitrile, pentadecanenitrile, and nonadecanenitrile.

Substrate specificity. The purified His-tagged enzyme was incubated with more than 50 different nitriles (5 mM each), and the relative activities determined by the quantitation of the amount of ammonia released. The enzyme showed a clear preference for fumarodinitrile (*trans*-1,2-dicyanoethene), which was converted with a >100-fold-higher relative activity than benzonitrile (Table 2). Also, some larger benzene derivatives, heterocyclic compounds, and long-chain aliphatic (di)nitriles were converted with higher relative activities than benzonitrile. In order to obtain a better estimation of the catalytic properties of the *Synechocystis* nitrilase, the basic catalytic constants were determined for some selected substrates. A comparison of the specificity constants (k_{cat}/K_m) (Table 3) confirmed the assumption that aliphatic dinitriles were the preferred substrates of the nitrilase.

Conversion of aliphatic dinitriles. It has been repeatedly shown that many nitrilases convert dinitriles preferentially to

TABLE 3. Kinetic constants of nitrilase with different substrates

Substrate	V_{max} (U mg ⁻¹)	K_m (mM)	K_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ M ⁻¹)
Benzonitrile	0.33	0.97	0.22	227
Indole-3-acetonitrile	1.5	2.4	1.0	417
2-Chloropropionitrile	1.4	5.2	0.92	177
Valeronitrile	0.7	4.4	0.47	107
Octanoic acid dinitrile	0.7	0.2	0.47	2,350
Decanoic acid dinitrile	0.4	0.3	0.27	900
Fumarodinitrile	109	28	72.7	2,600

mononitrile-monocarboxylic acids (1, 6, 12, 19). We therefore tested whether this was also valid for the nitrilase from *Synechocystis* sp. strain PCC6803. The purified enzyme was incubated with fumarodinitrile, succinodinitrile, adipodinitrile, 3-hexenoic acid dinitrile, octanoic acid dinitrile, or decanoic acid dinitrile (1 or 3 mM, each), and the time-dependent formation of ammonia was analyzed. The results suggested that the reactions stopped with all dinitriles with fewer than six carbon atoms after the release of 1 mol of ammonia per mol of the respective substrates. In contrast, 2 mol of ammonia were formed from 1 mol of octanoic acid dinitrile or decanoic acid dinitrile (Fig. 3).

Stability of the nitrilase in the presence of organic solvents.

The analysis of the substrate specificity of the nitrilase from *Synechocystis* sp. strain PCC6803 demonstrated that long-chain aliphatic compounds and rather hydrophobic aromatic compounds such as 2-naphthalenecarbonitrile were preferentially hydrolyzed. The enzymatic conversion of hydrophobic compounds in aqueous solutions can be limited by the low bio-availability of the hydrophobic substrates. It has been demonstrated that these limitations might be overcome by the inclusion of organic solvents into the medium (for recent reviews about the effects of organic solvents on enzymatic reactions, see reference 4 and 14). To the best of our knowledge, there is as yet only one study available in the literature which demonstrated that a bacterial nitrilase (from a pseudomonad) acted in the presence of organic solvents (23). Because the nitrilase from *Synechocystis* seemed to possess a preference for hydrophobic substrates, it appeared possible that the conversion of this type of compounds could be further increased by the addition of organic solvents. Therefore, the tolerance of the enzyme against organic solvents was tested, and it was found that the enzyme was active in the presence of a wide range of organic solvents (Fig. 4).

Conversion of long-chain aliphatic nitriles in the presence of organic solvents.

The conversion of (liquid) aliphatic nitriles with different chain lengths (4 to 19 carbon atoms) was analyzed in the presence of different concentrations of methanol, which were tolerated by the enzyme (0 to 20% [vol/vol]). These experiments demonstrated that the addition of methanol indeed resulted in increased conversion rates for aliphatic nitriles with chain lengths of more than 12 carbon atoms and that this effect most pronounced with dodecanoic acid nitrile. Thus, within 30 min in the presence of 10% (vol/vol) methanol, approximately twice the amount of ammonia was released compared to the control without added methanol. In contrast, no enhancement of the reaction rates was observed with aliphatic nitriles with shorter chain lengths or if the concentration

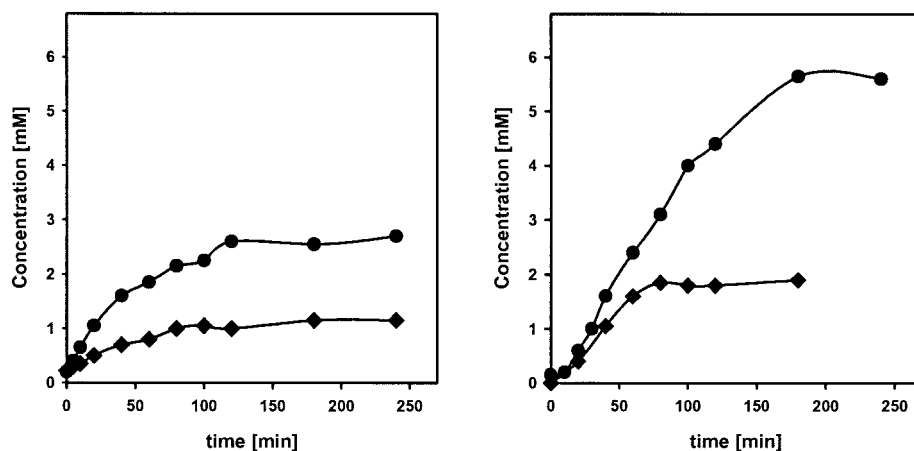


FIG. 3. Hydrolysis of fumarodinitrile and decanoic acid dinitrile by the purified nitrilase from *Synechocystis* sp. strain PCC6803. The reactions were performed in 1 ml of Na-K phosphate buffer (pH 7.0) containing 1 mM (◆) or 3 mM (●) fumarodinitrile (left panel) or decanoic acid dinitrile (right panel) and 80 μ g of purified enzyme. Aliquots (100 μ l each) were removed after different time intervals, the reactions were terminated by the addition of 10 μ l of HCl (1 M), and precipitated protein was removed by centrifugation (2 min, 16,600 \times g). The concentration of ammonia was subsequently determined by using the Spectroquant test.

of the organic solvent was increased to 40% (vol/vol) methanol.

The turnover of the aliphatic nitriles with different chain lengths was also analyzed in two-phase systems with hexane or heptane as organic phases (50% [vol/vol] each). No increase in the conversion rates of dodecanoic acid nitrile could be observed under these conditions.

Effects of organic solvents on the conversion of naphthalenecarbonitrile. Finally, the influence of organic solvents on the conversion of almost water-insoluble solid nitriles was tested with naphthalenecarbonitrile as the substrate. In this system, an increase in the reaction rates was observed in the presence of methanol and a slightly more pronounced effect in two-phase systems with water and hexadecane. The analysis of different ratios of hexadecane and the aqueous phase demonstrated that the maximal conversion of naphthalenecarbonitrile was achieved in the presence of 40% (vol/vol) hexadecane (Fig. 5).

DISCUSSION

We attempted here for the first time to obtain a novel bacterial nitrilase from the sequence information generated by various sequencing projects currently performed on bacterial genomes. The search for DNA sequences with sequence homologies to known nitrilases suggested that genes homologous to known nitrilases might be present in different microorganisms but that they are much less widespread compared, for example, to amidase genes. The successful cloning of a functional nitrilase demonstrated that this approach is potentially useful but also demonstrated the pitfalls of this strategy, especially that no selection takes place in order to obtain enzymes with high specific activities and that even screening of a huge spectrum of putative substrates will not result in a conclusive identification of the natural substrate(s) of the enzymes. In a different approach in order to obtain novel nitrilases without the traditional enrichment strategies, it was recently shown that active nitrilases can also be obtained from genomic libraries

constructed from the "metagenome" of (uncultured) microorganisms by expression cloning (5).

The present study clearly demonstrated that the cyanobacterium *Synechocystis* sp. strain PCC6803 harbors a gene that encodes a nitrilase activity, which could allow the organism to grow with various nitriles as nitrogen sources. Cyanobacteria are able to use some simple organic nitrogen compounds, such as amino acids, purines, urea, or acetamide, as nitrogen sources (7, 13, 28) but, to the best of our knowledge, there is no published information that describes the utilization of nitriles by cyanobacteria. Furthermore, the genetic organization of the nitrilase gene within the chromosome of *Synechocystis* spp. PCC6803 also did not suggest a putative natural function of the nitrilase because the gene was surrounded by two open reading frames, which encoded hypothetical proteins of unknown function, and the gene was not included in any obvious operon structure. Thus, we are currently unable to suggest any physiological function of the nitrilase in this microorganism, but it may be worthwhile to test whether *Synechocystis* sp. strain PCC6803 can utilize some of the nitriles that are substrates of the nitrilase as nitrogen sources.

The fundamental enzyme characteristics that have been determined for the nitrilase from *Synechocystis* sp. strain PCC6803 clearly resemble the data obtained with nitrilases from mesophilic chemotrophic bacteria: thus, similar temperature optima (\sim 40 to 45°C) and pH optima (pH 7 to 7.5) have been described for the nitrilases from *R. rhodochrous* J1 or *Alcaligenes faecalis* JM3 (16, 27). The observed substrate specificity of the *Synechocystis* nitrilase suggested that the enzyme most closely resembled the previously described group of aliphatic nitrilases, although the ability to hydrolyze aromatic substrates puts the enzyme somehow outside the established classification system for nitrilases. Previously, three bacterial nitrilases with a preference for aliphatic substrates have been described from *Acidovorax facilis* 72W, *Comamonas testosteroni*, and *R. rhodochrous* K22 (8, 18, 19, 24). Unfortunately, there is only very limited information available regarding the substrate specificities of these reference enzymes, but it is striking

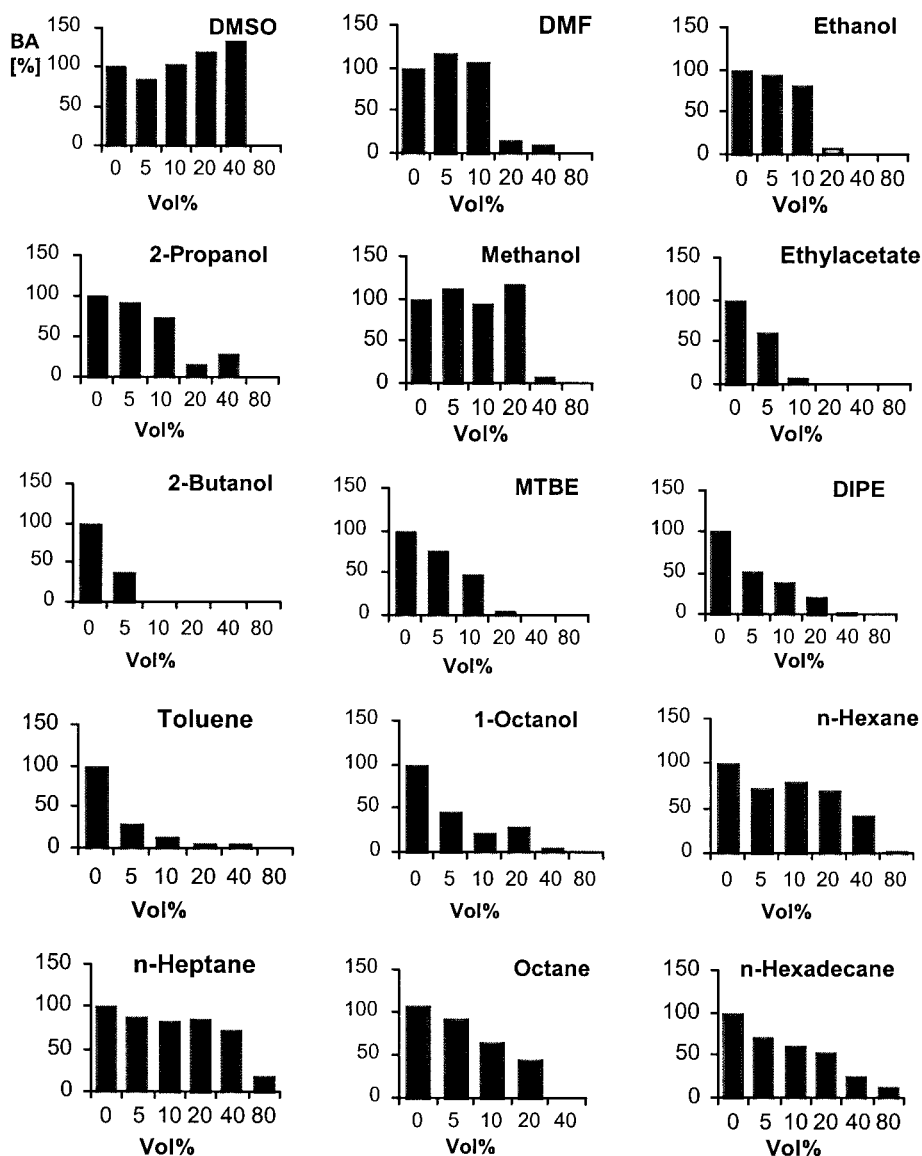


FIG. 4. Hydrolysis of benzonitrile by the purified nitrilase from *Synechocystis* sp. strain PCC6803 in the presence of different concentrations of various organic solvents. The reaction mixtures contained 19 μg of the purified (His-tagged) nitrilase in 250 μl of 50 mM Na-K phosphate buffer (pH 7.0) plus 0 to 200 μl of the solvents indicated. These enzyme preparations were incubated for 10 min at room temperature. Subsequently, benzonitrile (10 mM) was added, and the reaction mixtures were shaken at 30°C. The reactions were stopped after 30 min by the addition of 10% (vol/vol) 1 M HCl. Precipitated protein was removed by centrifugation (2 min, 16,000 \times g), and the concentration of benzoate formed was determined in the aqueous phase by HPLC. The enzyme preparation formed under these conditions in a purely aqueous phase 0.7 mM concentration of benzoate (100%). The relative amounts of benzoate (BA) formed in correlation to the control experiment in a purely aqueous phase are presented. DMSO, dimethyl sulfoxide; DMF, dimethyl formamide, MTBE, methyl *tert*-butyl ether; DIPE, diisopropyl ether.

ing that fumarodinitrile was identified as the preferred substrate for the nitrilase from *Synechocystis* sp. strain PCC6803 and also for the aliphatic nitrilase from *Acidovorax facilis* strain 72W (8), although the nitrilase from *Synechocystis* was clearly differentiated by its ability to convert substrates such as benzylcyanide, butyronitrile, crotonitrile, or valeronitrile from the *Acidovorax* nitrilase. Only the nitrilase from *R. rhodochrous* K22 has been purified and biochemically characterized in greater detail from the enzymes that have been previously described as aliphatic nitrilases (18). This enzyme demonstrated with almost all substrates rather low specific activities

(<1 U/mg of protein) and reached the highest specific activities (3.3 U/mg of protein) with glutaronitrile (18). In contrast, for several aromatic nitrilases and arylacetone nitrilases, specific activities of 15 to nearly 150 U/mg of protein have been described for the conversion of specifically favored substrates (10, 16, 27, 40). The observation that the purified *Synechocystis* nitrilase demonstrated a V_{max} of >100 U/mg of protein with its preferred substrate fumarodinitrile demonstrated that nitrilases can also convert aliphatic substrates with rather high catalytic efficiencies.

In order to predict a putative natural function for the ni-

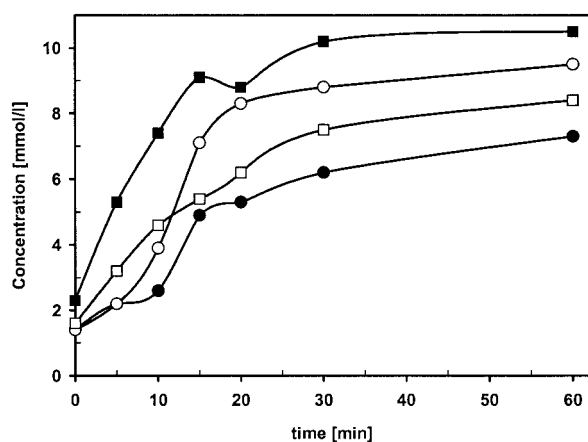


FIG. 5. Conversion of naphthalenecarbonitrile by the purified nitrilase from *Synechocystis* sp. strain PCC6803 in the presence of different volumes of hexadecane. The reaction mixtures contained 580 μ g of the purified (His-tagged) nitrilase in 200 μ l of 50 mM Na-K phosphate buffer (pH 7.0) plus 10 mM naphthalenecarbonitrile. Different volumes of hexane were added (0% [vol/vol] (●), 20% [vol/vol] (○), 40% [vol/vol] (■), or 60% [vol/vol] (□)), and the reaction mixtures were shaken at 30°C in a Thermomixer at 1,000 rpm. The reactions were stopped after different time intervals by the addition of 10% (vol/vol) 1 M HCl. Precipitated protein was removed by centrifugation (2 min, 16,000 \times g), and the concentration of ammonia formed was determined in the aqueous phase by using the Spectroquant ammonia test.

trilase, sequence comparisons were performed with various known or predicted nitrilases from microbial or plant origin. These alignments demonstrated that the known bacterial aliphatic nitrilases did not form a distinct cluster within the nitrilase group. Obviously, the nitrilase from *Synechocystis* sp. strain PCC6803 did not cluster tightly with the known microbial nitrilases but was placed in the dendrograms somewhere

intermediately between the bacterial and plant nitrilases (Fig. 6). Many plants (especially members of the *Cruciferae*) encode for several different nitrilases, and for *Arabidopsis thaliana* four different nitrilase genes have been identified. Three of these genes (*Nit1* to *Nit3*) demonstrated high sequence similarities with each other, and the encoded nitrilases are able to convert indole-3-acetonitrile to the plant growth hormone indole-3-acetic acid. These three nitrilases also show a rather similar substrate specificity (39). In contrast, the fourth nitrilase (*Nit4*) differed according to its sequence and substrate specificity significantly from the three others, and it has been recently suggested that *Nit4* and its orthologs in other plants encode for a β -cyano-L-alanine hydratase/nitrilase that is involved in cyanide detoxification in plants (31). Surprisingly, the *Synechocystis* nitrilase did not convert β -cyano-L-alanine (unpublished results), although it hydrolyzed the C-4 compounds fumarodinitrile with extremely high specific activities. Some interesting parallels could be found when the substrate specificity of the *Synechocystis* nitrilase was compared to the *Nit1* nitrilase from *Arabidopsis thaliana* (29). Thus, both enzymes demonstrated a certain preference for long-chain aliphatic nitriles. Furthermore, both enzymes converted dinitriles with higher relative activities compared to the mononitriles of the same chain length and almost did not hydrolyze nitriles that carried in the α -position larger substituents (e.g., 2-phenylpropionitrile). Nevertheless, several differences between the two enzymes were also observed. For example, the nitrilase from *Synechocystis* converted benzonitrile and indole-3-acetonitrile with approximately the same or even higher relative activities compared to butyronitrile, whereas these substrates were only converted by the *Arabidopsis* nitrilase with <3% of the activity observed with butyronitrile (29).

In conclusion, it appears that the *Synechocystis* nitrilase hydrolyzes the broadest range of nitriles from all nitrilases currently known and that thus the approach used to obtain this

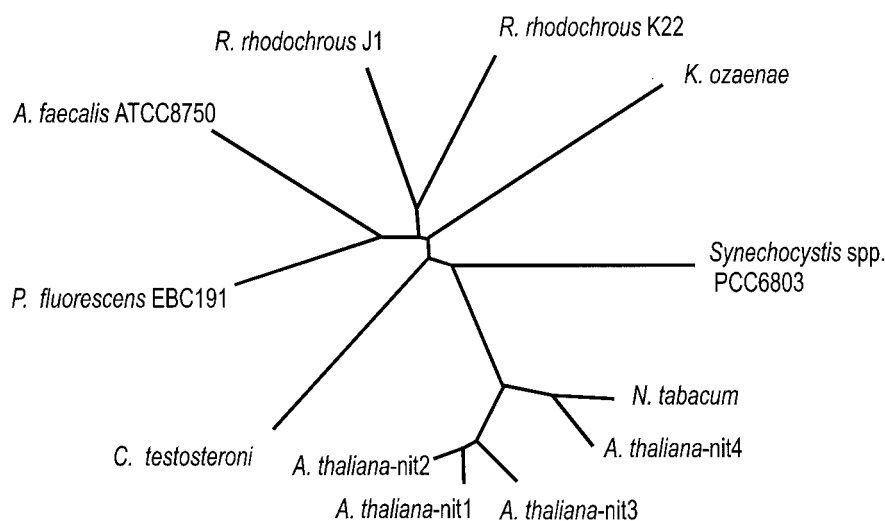


FIG. 6. Dendrogram resulting from pairwise alignments of amino acid sequences of different nitrilases. The following sequences with their relevant NCBI registration numbers were used: *Alcaligenes faecalis* JM3 (D13419); *C. testosteroni* (L32589); *Klebsiella pneumoniae* (*ozaenae*) (J03196); *Pseudomonas fluorescens* EBC 191 (C. Kiziak, unpublished data); *R. rhodochrous* K22 (D12583); *R. rhodochrous* J1 (D12583); *Synechocystis* sp. strain PCC 6803 (D64005); *Nit4* from *Arabidopsis thaliana* (U09961) and *Nicotina tabacum* (D63331); and *Nit1*, *Nit2*, and *Nit3* from *Arabidopsis thaliana* (Y07648). The sequences were aligned by using the program CLUSTAL X1.8, the dendrograms calculated by bootstrap neighbor joining, and the phylogenetic tree drawn by using the program TreeView 1.6.6, with the standard parameters.

nitrilase indeed resulted in the aspired isolation of a novel type of nitrilase.

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