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A cyanase is transcriptionally regulated by arginine and involved in cyanate decomposition in *Sordaria macrospora*

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ABSTRACT

Cyanase degrades toxic cyanate to NH_3 and CO_2 in a bicarbonate-dependent reaction. High concentrations of cyanate are fairly toxic to organisms. Here, we characterize a eukaryotic cyanase for the first time. We have isolated the *cyn1* gene encoding a cyanase from the filamentous ascomycete *Sordaria macrospora* and functionally characterized the *cyn1* product after heterologous expression in *Escherichia coli*. Site-directed mutagenesis confirmed a predicted catalytic centre of three conserved amino-acids. A Δcyn1 knockout in *S. macrospora* was totally devoid of cyanase activity and showed an increased sensitivity to exogenously supplied cyanate in an arginine-depleted medium, defects in ascospore germination, but no other obvious morphological phenotype. By means of real-time PCR we have demonstrated that the transcriptional level of *cyn1* is markedly elevated in the presence of cyanate and down-regulated by addition of arginine. The putative functions of cyanase in fungi are discussed.

1. Introduction

Cyanase (also known as cyanate lyase; EC 4.2.1.104) converts cyanate to CO_2 and NH_3 in a bicarbonate-dependent reaction: $NCO^- + HCO_3^- + 2H^+ \rightarrow 2CO_2 + NH_3$ (Johnson and Anderson, 1987). The enzyme was first discovered in *Escherichia coli* (Taussig, 1960). BLAST searches revealed that genes homologous to the gene-encoding cyanase in *E. coli* are present in genomes of various proteobacteria, cyanobacteria, plants and fungi (Guilloton et al., 2002). Despite the availability of a detailed characterization of its activity in *E. coli* and the cyanobacterium *Synechococcus* sp., the physiological role of cyanase is not exactly understood (Anderson and Little, 1986; Miller and Espie, 1994). Marked differences in cyanase-mediated cyanate metabolism among species have been documented. For example, in bacteria cyanase has an important role in cyanate detoxification and cyanate-dependent production of NH_3 as an alternative nitrogen source (Anderson et al., 1990; Kunz and Nagappan, 1989), as well as in the production of CO_2 for carbon utilization in photosynthetic cyanobacteria (Espie et al., 2007).

X-ray crystallography has proved that the *E. coli* cyanase is active as a homodecamer of 17-kDa subunits and identified three amino-acids in each subunit as the catalytic residues that bind to cyanate and bicarbonate (Walsh et al., 2000). Kinetic analyses have shown that cyanase catalysis leads to the formation of a putative dianion intermediate. Subsequently, decarboxylation leads to the production of a first CO_2 molecule and one molecule of carbamate. The latter decomposes in a spontaneous decarboxylation process into a second CO_2 plus NH_3 (Anderson, 1980; Kozliak et al., 1995).

In the environment, cyanate is formed as a consequence of spontaneous dissociation of urea in aqueous solution (Dirnhuber and Schutz, 1948) and photo-oxidation of cyanide (Raybuck, 1992). Moreover, cyanate accumulates during the industrial removal of highly toxic cyanide from mining wastewater by a permanganate oxidation reaction leading to the production of lesser toxic cyanate (Sancho et al., 2005). Within living organisms, cyanate results from the non-enzymatic decomposition of carbamoyl phosphate (Suzuki et al., 1996). The toxicity of cyanate probably results from its reactivity with functional groups of proteins (Stark, 1965).

To date, eukaryotic cyanases have not been characterized, and their functional role is completely unknown. In the present study, we have identified a cyanase-encoding gene in the filamentous fungus *Sordaria macrospora*, which belongs to the order of Sordariales within the class of ascomycetes and has long been used as a model organism of fruiting body development (Lundqvist, 1972; Masloff et al., 1999; Nowrousian et al., 1999; Pöggeler et al., 2006a). The *S. macrospora* cyanase, which was heterologously expressed in *E. coli*, is highly active *in vitro* and catalyses the decomposition of cyanate in a bicarbonate-dependent manner. Site-directed mutagenesis revealed that the catalytic centre of

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the cyanase is conserved from bacteria to fungi. A *S. macrospora* Δcyn1 knockout mutant cannot convert exogenously supplied cyanate to CO_2 and NH_3 and was shown to be more sensitive to cyanate than the wild-type when vegetatively grown on cyanate containing medium. The vegetative growth defect caused by cyanate can be partially complemented by the addition of arginine in both, the mutant and the wild-type strain. Quantitative real-time PCR analysis revealed that *cyn1* is up-regulated by cyanate and down-regulated by arginine. Furthermore, we observed that the mutant strain shows a decreased ascospore-germination rate.

2. Materials and methods

2.1. Strains and culture conditions

Escherichia coli strain SURE (Stratagene) was used as the bacterial host strain for plasmid amplification. Cloning and propagation of recombinant plasmids was done under standard conditions (Sambrook et al., 2001). Fungal strains used in this study are summarized in Table 1. *S. macrospora* strains were cultivated on corn-meal medium, on SWG minimal medium or in liquid CM medium (Esser, 1982; Nowrousian et al., 1999). Growth velocities were determined according to the method of Nowrousian and Cebula, (2005) and mycelial growth was monitored by the dry cell weight according to Nolting and Pöggeler (2006). The determination of the germination efficiency was conducted by isolating at least 100 single ascospores, and by counting germinated spores after 1, 2 and 3 days of incubation on corn-meal medium supplied with 0.5% of sodium acetate and with or without 10 mM of potassium cyanate.

2.2. Transformation of S. macrospora

Transformation of *S. macrospora* was performed as described by Nowrousian et al. (1999). Fungal protoplasts were transformed either with linear PCR fragments generated from plasmid pCYN1-KO, as co-transformation with plasmids pDNat1 and pCYN1-Compl or with plasmid pGPD-CYN1 (Table 2). Transformants were selected on either hygromycin B (110 U/ml) or nourseothricin (50 μg/ml) containing CM medium (Walz and Kück, 1995).

2.3. Preparation of nucleic acids, hybridization protocols and PCR

Isolation of *S. macrospora* genomic DNA was carried out as described by Pöggeler et al. (1997b). Southern blotting and hybridization were performed according to standard techniques (Sambrook et al., 2001), using ^{32}P-labeled DNA probes. PCR amplification was carried out with the Expand Long Template PCR polymerase kit (Roche) following the manufacturers protocol.

Total RNA was isolated as described by Fröhlich et al. (2005) with the following modification: frozen mycelial samples supplemented with 1 ml Trizol (Invitrogen) were homogenized two times (2 min, 1000 Hz) using the TissueLyser (Qiagen).

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously (Pöggeler et al., 2006b). The following modifications have been done: reverse transcription was performed using 400 U Superscript III reverse transcriptase (Invitrogen) and Oligo$(dT)_{20}$ Primer. Real-time PCR was carried out with a Mastercycler® ep *realplex* (Eppendorf). Amplification of a part of the SSUrRNA with primers SSU-f and SSU-r (Supplementary Table S1) was used as a reference for normalization of Ct values.

2.5. Sequence analysis

The *cyn1* gene is located upstream of the *ppg2*-gene and was identified by sequencing a cosmid clone previously shown to contain the pheromone precursor gene *ppg2* (Pöggeler, 2000). DNA sequencing was performed by MWG Biotech Customer Service (Ebersberg, Germany) or by G2L-sequencing service of the Göttinger Genom Labor (Georg-August University Göttingen, Germany). Nucleotide sequence of the *cyn1* gene was deposited in the EMBL database under the following accession number: AM773726.

Protein sequence data of cyanase genes of other organisms were obtained from the public databases NCBI Entrez (http://www.ncbi.nlm.nih.gov/entrez/), or by BLAST-P searches (Altschul et al., 1997) of the fully sequenced genomes at the BROAD INSTITUTE (http://www.broad.mit.edu/annotation/fungi/fgi/). Multiple protein sequence alignments were performed using the ClustalX program (Thompson et al., 1997). The prediction of promoter elements was done by using different "Hamming–Clustering" methods (http://www.itb.cnr.it/sun/webgene//) and the "Promoter Predictor" (http://www.fruitfly.org/seq_tools/promoter.html). Protein secondary-structure prediction was done with the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) (Jones, 1999). Conserved protein domains were predicted using the SUPERFAMILY database at http://supfam.org/SUPERFAMILY/ (Gough et al., 2001).

2.6. Phylogenetic analysis

Phylogenetic analyses were made with programs contained in the program package PHYLIP version 3.6 (http://evolution.genetics.washington.edu/phylip.html). The number of amino-acid replacements per sequence position separating each pair of sequences was estimated using the PAM option of the PROTDIST program and were then used for constructing phylogenetic trees with the neighbor-joining program NEIGHBOR. Statistical significance was evaluated by bootstrap analysis, with 1000 iterations of bootstrap samplings and reconstructing of trees by NEIGHBOR. A majority rule consensus tree was subsequently generated using the program CONSENSE.

2.7. Heterologous expression of tagged protein products

A cDNA of the *cyn1* gene was generated by RT-PCR with primer pair phesa17/phesa18 (Table S1) as described previously (Pöggeler

Table 1
Sordaria macrospora strains used in this study

Strain	Genotype and phenotype[a]	Source
S17736	Wild-type	Lab collection[b]
T11-3-1-3	Primary transformant, heterokaryotic, hyg$^+$	This study
SE7 (Δcyn1)	Single-spore isolate, hyg$^+$	This study
Δcyn1:cyn1^{ect}	Complementation strain carrying ectopic copy of *cyn1*, hyg$^+$, nat$^+$	This study
Δcyn1:gpd-cyn1^{ect}, T1-T3	Complementation strain carrying an ectopic copy of *cyn1* under control of the *A. nidulans gpd* promoter, hyg$^+$, nat$^+$	This study

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