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A novel gene encoding amidinotransferase in the cylindrospermopsin producing cyanobacterium *Aphanizomenon ovalisporum*

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Abstract

The hepatotoxin cylindrospermopsin is produced by several cyanobacteria species, which may flourish in tropical and sub-tropical lakes. Biosynthesis of cylindrospermopsin is poorly understood but its chemical nature, and feeding experiments with stable isotopes, suggested that guanidinoacetic acid is the starter unit and indicated involvement of a polyketide synthase. We have identified a gene encoding an amidinotransferase from the cylindrospermopsin producing cyanobacterium *Aphanizomenon ovalisporum*. This is the first report on an amidinotransferase gene in cyanobacteria. It is likely to be involved in the formation of guanidinoacetic acid. The *aoaA* is located in a genomic region bearing genes encoding a polyketide synthase and a peptide synthetase, further supporting its putative role in cylindrospermopsin biosynthesis.

Keywords: Amidinotransferase; *Aphanizomenon*; Cylindrospermopsin; Toxic cyanobacterium; Polyketide synthase

1. Introduction

Cylindrospermopsin is a water-soluble hepatotoxin. It is produced by several cyanobacteria species including *Aphanizomenon ovalisporum*, *Cylindrospermopsis raciborskii, Anabaena bergii* and *Umesakia natans* [1,2]. These cyanobacteria may flourish in tropical and sub-tropical regions around the world and therefore, their blooms constitute a health hazard. The role of specific residues essential for cylindrospermopsin toxicity is emerging [3]. Apart from cylindrospermopsin toxicity primarily to the liver, kidney and other organs, carcinogenic activity due to the presence of the uracil and sulfinated guanidino moieties was recently suggested [4,5].

The mechanism of cylindrospermopsin biosynthesis was partly resolved by following cylindrospermopsin production in *C. raciborskii* cultures that were supplemented with stable isotopes of ^{13}C, ^{18}O, ^{15}N and ^{3}H [6]. The feeding experiments suggested that five molecules of acetate are catalytically condensed by a polyketide synthase (PKS) to form the carbon skeleton of cylindrospermopsin. Two other carbons and one of the nitrogens of the guanidine moiety are donated by glycine. Guanidinoacetic acid was proposed as a source for two nitrogen atoms of the guanidino moiety and as a starter unit that initiates the biosynthetic process. The guanidinoacetic acid is probably formed by amidination of glycine, but the exact origin of the other N atoms in the guanidino moiety is not yet resolved [6]. Formation of guanidinoacetic acid may be catalyzed by amidinotransferase. The latter transfers the amidino group from L-arginine to glycine during creatine biosynthesis in vertebrates; to inosamine phosphate for the formation of streptomycin by *Streptomyces griseus*; and to lysine during phaseolotoxin production in *Pseudomonas syringae* [7–9]. Another component of the cylindrospermopsin structure, the polyketide, is presumably catalyzed by a PKS. This is a family of multienzyme complexes which contain all the enzymatic activities re-

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quired for the sequential decarboxylative condensation of short acyl units, analogous to the chain elongation step of fatty acid biosynthesis. The PKS contains all the following active sites: β-keto acyl synthase (KS), acyl transferase (AT), acyl carrier protein, dehydrogenases (DH) and the 'swinging arms' of the phosphopantetheine binding domain (PPB) [10]. These enzymes, responsible for the formation of polyketides, are involved in the biosynthesis of a broad family of compounds including different antibiotics and cyanobacterial toxins [1,11].

In this study, we have identified a novel gene, designated *aoaA*, encoding amidinotransferase in the cylindrospermopsin producing cyanobacterium *A. ovalisporum*. The *aoaA* was found within a genomic region encoding PKS and peptide synthetase, indicating its potential role in cylindrospermopsin biosynthesis.

2. Materials and methods

2.1. Growth conditions

A toxic strain of *A. ovalisporum* was isolated from Lake Kinneret [12]. In this study, we first obtained an axenic culture using the methods developed by Prof. P. Wolk [13]. The axenic cultures were grown on a shaker (100 rpm) in 500-ml flasks containing medium BG11 [14] supplemented with 20 mM TAPS–NaOH buffer, pH 9 and 10 mM $NaHCO_3$. Light intensity was 25 μmol photons m^{-2} s^{-1}, provided by cool white fluorescent lamps, temperature was 30°C.

2.2. DNA extraction

The cells were collected by centrifugation, washed with TE and resuspended in 300 μl lysozyme buffer (20 mM EDTA, 150 mM NaCl, 15 mg lysozyme) at 37°C, for 3 h, followed by addition of lysis buffer (100 mM NaCl, 500 mM Tris–HCl, 5% SDS). After three freeze–thaw cycles, proteinase K was provided (final concentration was 100 μg ml^{-1}) and incubation continued for 30 min at 37°C. The DNA was then extracted with phenol, phenol:chloroform and chloroform and subsequently precipitated by the addition of 2.5 M Na-acetate and an equal volume of cold isopropanol at −20°C for 30 min. After centrifugation the DNA was washed with cold 70% ethanol, dried and resuspended in sterile water. Further DNA techniques were carried out according to standard procedures [15].

2.3. Preparation of a DNA library from A. ovalisporum

A genomic DNA library was raised by cloning size-fractionated (7–10 kb), partially *Sau*3A digested DNA, within a dephosphorylated (SAP, Boehringer-Mannheim) *Bam*HI site of plasmid pUC118. Transformation to Epicurian *Escherichia coli* XL10-Gold ultracompetent cells (Stratagene), according to the manufacturer's instructions and selection on LB plates with 50 μg ml^{-1} carbamecylin. The plasmids were extracted by a Miniprep kit (Qiagen) according to the manufacturer's instructions.

2.4. Identification and cloning of a keto acyl synthase (KS)

A genomic DNA fragment bearing conserved region of KS was amplified by polymerase chain reaction (PCR) using the following degenerate primers:

5′-GGRTCNCCIARYTGIGTICCIGTICCRTGIGC-3′ (forward) and

5′-MGIGARGCIYTIGCIATGGAYCCICARCARM-G-3′ (reverse) [16]. The PCR protocol was: 94°C 5 min, 40 cycles of (94°C 1 min, 57°C 1.5 min, 72°C 1 min), 72°C 5 min, 10°C (hold). A fragment of 650 bp was synthesized, cloned and sequenced and showed high similarity to KS in a BLAST search (see Fig. 1). The KS fragment was labeled with [^{32}P]dCTP by random priming and used as probe to screen the genomic library of *A. ovalisporum* by colony hybridization. Three colonies were selected for further analysis including subcloning, purification of DNA and plasmids [15] and sequencing and mapping. Additionally, the KS fragment and the amidinotransferase identified here were used as probes for Southern hybridization, using a non-radioactive probe (NEN) according to the manufacturer's instructions, at 65°C.

2.5. Sequence analysis

Sequence analysis was performed using the ABI 377 Prism DNA sequencer. Putative amino acid sequences encoded by the open reading frames found here were compared with those in the databases through the NCBI website (www.ncbi.nlm.nih.gov/blast/Blast). Multiple alignments were performed with the aid of the program CLUSTALW.

3. Results and discussion

The polyketide nature of cylindrospermopsin and the feeding experiments [6] suggested that a PKS of either type I or II is involved in its assembly. A 650-bp fragment was amplified from the genomic DNA of *A. ovalisporum* using degenerate primers designed according to a conserved region of KS. The sequence of this fragment showed high homology to the KS regions within PKSs in various bacteria and fungi (Fig. 1). Screening of the genomic library of *A. ovalisporum* by colony hybridization, using the 650-bp fragment as a probe, revealed several different positive colonies. Three of them, designated j2b, j1a and j1c, were selected for further analysis.

Sequence analysis of clone j2b (included in accession number AF395828) revealed a 1177-bp fragment highly homologous to genes encoding amidinotransferases in var-

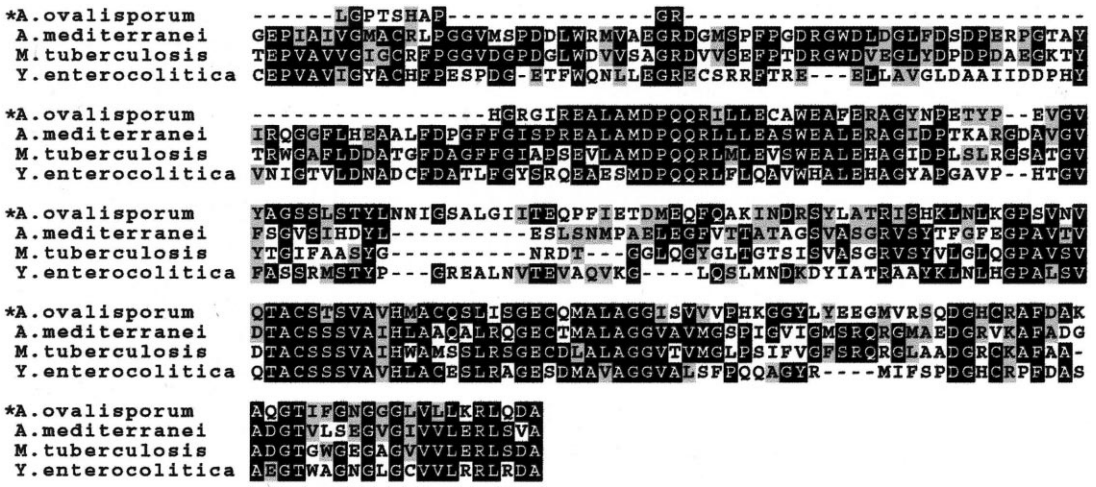

Fig. 1. Alignment of the 650-bp conserved region encoding KS from *A. ovalisporum* (indicated by *) with sequences identified from *Mycobacterium tuberculosis* (AAA50929), *Yersinia enterocolitica* (CAA73127) and *Amycolaptosis mediterranei* (AAC01711). Black background, identical residues; gray background, similar residues.

ious organisms (Fig. 2). This gene was designated *aoaA* for *Aphanizomenon ovalisporum* amidinotransferase A. This is the first report on the presence of an amidinotransferase encoding gene in cyanobacteria. We predict that it is also present in other cylindrospermopsin producing species.

There are two structurally well characterized amidinotransferases: the prokaryotic one from *S. griseus*, involved in the formation of streptomycin, and the eukaryotic type involved in the creatine biosynthetic pathway [7,8]. In these enzymes, structural differences were correlated with variations in the amidino acceptor, inosamine phosphate or glycine in the prokaryotic and eukaryotic forms, respectively. The structure of amidinotransferase and the nature of the amidino acceptor in *A. ovalisporum* are not yet resolved, but sequence comparisons showed higher similarity of this prokaryotic-originated gene to the eukaryotic type.

Genomic DNA was hybridized sequentially to two

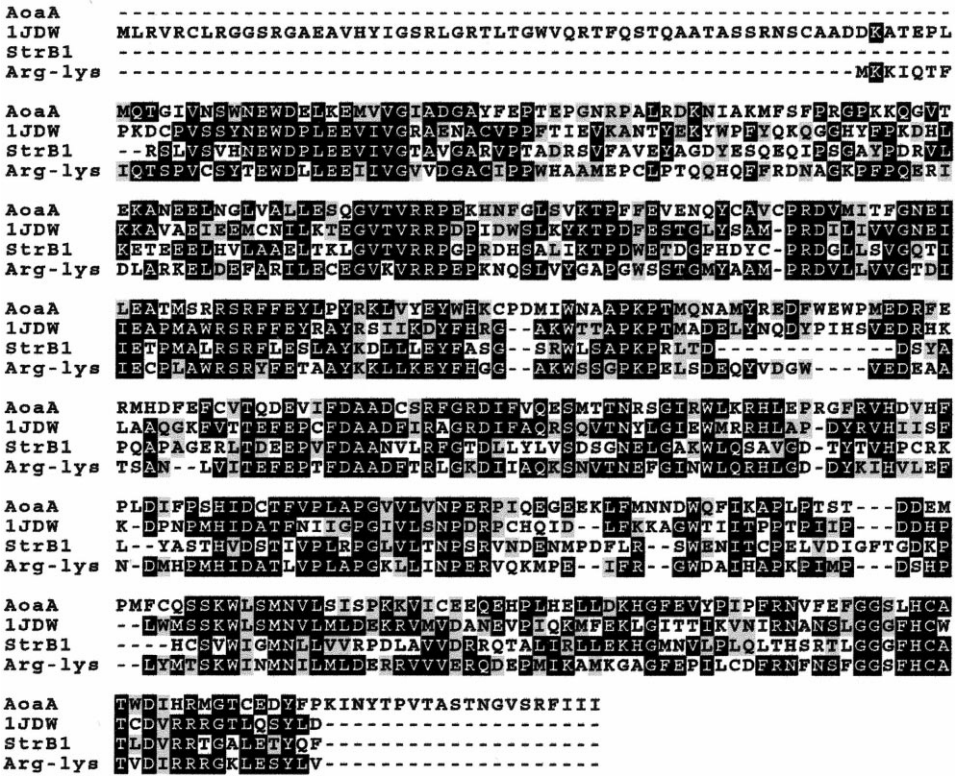

Fig. 2. Sequence comparison of the amidinotransferase (AoaA) from *A. ovalisporum* with human L-arginine:glycine amidinotransferase (1JDW, accession number 2914341 [20]), L-arginine:lysine amidinotransferase from *P. syringae* pv. Phaseolicola (Arg–Lys, accession number AAD56249 [9]) and L-arginine:inosaminephosphate from *S. griseus* (StrB1, accession number 4389220 [7]). Black background, identical residues; gray background, similar residues.

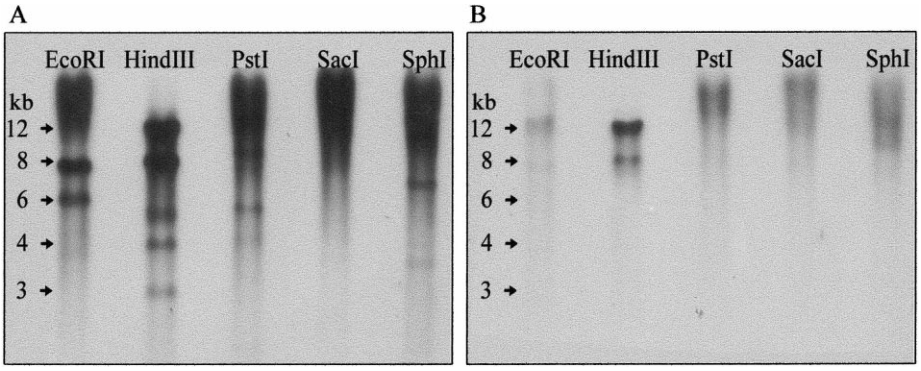

Fig. 3. Southern analyses of *A. ovalisporum* DNA digested with several enzymes. A: The 650-bp bearing part of β-keto-acyl synthase was used as a probe. B: A 492-bp fragment containing part of *aoaA* was used as a probe. The same membrane was used in A and B. Note that both probes hybridized to the 12-kb fragment.

probes, the 650-bp fragment bearing part of the KS and a 492-bp fragment containing part of *aoaA* (from position −70 to 422 in the coding region), using the same membrane (Fig. 3). The Southern analysis showed that the genomic region bearing conserved KS motifs extends to about 35 kb, as revealed from the sum of the fragments in the *Hin*dIII digest (Fig. 3A). Both probes hybridized to a 12-kb fragment (Fig. 3B) suggesting that the *aoaA* might be located within the PKS region. This was confirmed by sequence analysis. The Southern analyses also showed a 8-kb *Hin*dIII fragment which hybridized with both *aoaA* and the conserved KS region for a reason not yet known.

In prokaryotes, genes involved in the same physiological process or biochemical pathway are often clustered. Figure 4 shows that genes encoding amidinotransferase, PKS and peptide synthetase are clustered in the same genomic region in *A. ovalisporum*, as revealed by sequence analysis (accession number AF395828). The conserved regions, indicated in Fig. 4, were identified on the basis of very high homology to identical motifs in other genes. Conserved domains found here include: KS, AT and DH. The *aoaA*, encoding the amidinotransferase, is located upstream of the PKS, on the reverse strand. The PKS found here bear several active sites within one reading frame, a characteristic of type I PKS. These are large multidomain proteins that carry all the active sites required for polyketide synthesis and are encoded by a single open reading frame. In type II, the active sites are distributed among several smaller polypeptides that are transcribed by different sequential genes [11]. Recently, partial PKS sequences from *C. raciborskii* and *A. bergii* were reported [2]. Downstream of *aoaA*, we found a peptide synthetase fused to a PKS bearing motif, encoding an AMP-binding domain (AMPB), the PPB, two KS domains and an AT domain. Some of these domains were also detected in genes encoding other cyanobacterial toxins such as the *mcy* genomic region encoding various types of microcystins in *Microcystis* [2,17–19].

This study identified genes encoding amidinotransferase, PKS and peptide synthetase in *A. ovalisporum*, all essential for cylindrospermopsin biosynthesis. Based on sequence homology and clustering of these genes at the same ge-

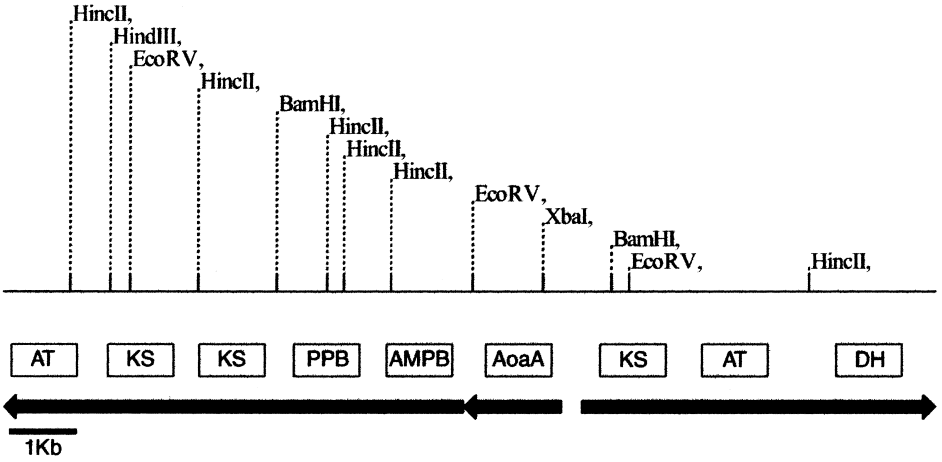

Fig. 4. A schematic map of the 11 354-bp genomic region in *A. ovalisporum* encoding PKS, amidinotransferase and a peptide synthetase fused to a PKS. The map was revealed by sequence analysis, accession number AF395828. The conserved regions included in this region are indicated by their initials and were identified as KS, AT, DH, AoaA, AMP-binding domain (AMPB), and a PPB as revealed by a blast CD search (www.ncbi.nlm.nih.gov/Structure/cdd/qrpsb.cgi). Sites where certain restriction enzymes could digest this region are indicated.

nomic region, it is likely that they participate in cylindrospermopsin formation. However, unequivocal evidence that this is indeed the case would have to rest on further analysis including gene inactivation (in progress).

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