The dspA gene product of the cyanobacterium Synechocystis sp. strain PCC 6803 influences sensitivity to chemically different growth inhibitors and has amino acid similarity to histidine protein kinases

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We have cloned and sequenced a gene of the cyanobacterium Synechocystis sp. strain PCC 6803 named dspA (encoding drug sensory protein A; DspA), mutations in which result in cross-resistance to the herbicides difunon and diuron, as well as to the calmodulin antagonists chlorpromazine and trifluoperazine. The dspA gene encodes a polypeptide of 663 amino acids with a predicted molecular mass of 74.5 kDa. The molecular nature of two mutations in the dspA gene leading to the cross-resistance has been determined. Targeted mutagenesis of the dspA gene was performed using a kanamycin-resistance gene cartridge. Resulting mutant strains were checked for resistance to difunon and chlorpromazine and showed cross-resistance to both agents. The C-terminal portion of the deduced amino acid sequence of DspA shares significant similarity with the conserved region of histidine protein kinases (HPKs). Hydrophobicity analysis of the amino acid sequence of DspA indicated the existence of two hydrophobic regions in the N-terminal portion that are characteristic of the bacterial sensory HPK family. We suggest that protein DspA is a HPK involved in chemical sensing.

Keywords: cyanobacteria, histidine protein kinase, herbicide resistance

INTRODUCTION

Adaptation responses in bacterial cells are often controlled by two families of signal transduction proteins: histidine protein kinases (HPKs) and response regulators (RRs) (Stock et al., 1989). A large number of the HPKs are membrane associated and function as membrane receptors, while the cytoplasmic RRs transduce information from the receptors to adaptive response elements, mediating changes in gene expression. A signal transduction occurs through the chain of phosphorylation and dephosphorylation reactions exerted by HPK and its associated RR. Two-component systems control a wide variety of processes including sporulation, transformation competence, chemotaxis, osmoregulation and metabolic adaptation to changes in nutrient sources (Stock et al., 1989, 1990). HPKs and RRs are found in many Gram-negative and Gram-positive bacteria (Stock et al., 1989; Parkinson & Kofoid, 1992). A number of genes encoding members of two-component systems have been found in cyanobacteria (Mann, 1994). HPKs and RRs involved in the adaptive response to phosphate limitation were identified in Synechococcus sp. strain PCC 7942 (Aiba et al., 1993). The PatA protein required for the differentiation of intercalary heterocysts in Anabaena sp. strain PCC 7120 has amino acid similarity to the Escherichia coli chemotactic response regulator CheY (Liang et al., 1992). RRs are also involved in the process termed complementary chromatic adaptation in the filamentous cyanobacterium Fremyella diplosiphon (Chiang et al., 1992). A number of other putative RRs (Chitnis et al., 1989; Schluchter & Bryant, 1992) as well as putative HPKs (Nagaya et al., 1993) have been discovered, but their functions are not clear at present.

Cyanobacteria provide a simple experimental system for...
the study of oxygen-evolving photosynthesis and many other metabolic processes that are similar to those of plants. Moreover, these processes are targets for many commercial herbicides. Herbicide-resistant mutants of cyanobacteria can be selected in great numbers and resistance-conferring genes can be easily isolated. A number of genes responsible for resistance to various herbicides have been cloned from some species of *Synechococcus* and *Synechocystis* (Golden & Haselkorn, 1985; Ajlani et al., 1989; Friedberg & Seijffers, 1990; Chamovitz et al., 1990, 1991; Martinez-Ferez & Vieque, 1992; Chesnavichene et al., 1994). The unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (hereafter called *Synechocystis* 6803) provides several advantages for such investigations. This organism has a naturally occurring genetic transformation system (Grigorieva & Shestakov, 1982). Transfected DNA molecules undergo homologous double reciprocal recombination with chromosomal DNA, which allows for directed gene replacement as well as for phenotypic complementation of defined mutant strains of *Synechocystis* 6803 (Williams, 1988).

Recently, we have isolated mutant strains of *Synechocystis* 6803 (DF1 and CPN6) that are cross-resistant to the herbicides difunon ([5-dimethylaminomethylene]-2-oxo-4-phenyl-2,5-dihydrofurancarbonitrite-(3)) and diuron [3-(4-dichlorophenyl)-1,1-dimethylurea] and the calmodulin antagonists chlorpromazine [2-chloro-10-(3-methylpyridin-1-yl)propyl-1,1-dimethylurea] and trifluoperazine (10-[3-(4-methylpipеразин-1-yl)propyl]-2-(trifluoromethylurea)] (Shestakov & Bartsevich, 1993). Difunon, as well as a number of other bleaching herbicides, blocks carotenogenesis (Urbach et al., 1976). Diuron blocks electron transport in photosystem II (Steinback et al., 1981). The calmodulin antagonists inhibit a variety of calcium-dependent processes (Onek & Smith, 1992).

Here, we describe the cloning and sequencing of a gene of *Synechocystis* 6803, the inactivation of which results in cross-resistance to these inhibitors. The product of this gene shares significant amino acid similarity with HPKs.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The wild-type (WT) strain of *Synechocystis* sp. strain PCC 6803 (from the Pasteur Culture Collection) and its mutants were grown in BG11 medium (Allen, 1968) at 30 °C under 60 μmol m⁻² s⁻¹ fluorescent light. Solid BG11 medium was supplemented with 1% (w/v) agar. The isolation of spontaneous herbicide-resistant mutant strains DF1 and CPN6 has been previously described by Shestakov & Bartsevich (1993).

*Escherichia coli* strain NM522 (supE thi Δ (lac-proAB) Δ(kdsMS-mcrB) (r6m*-m12) (F' proAB lacZAM15) (Gough & Murray, 1983), plasmid vector pUC19 and phage vectors M13mp18 and M13mp19 (Yanisch-Perron et al., 1985) were used for gene cloning and sequencing. The kanamycin-resistance (*Km*') gene cartridge from pUC4K (Vieira & Messing, 1982) was used to create insertion mutants. Plasmids pDF1, pCPN6, pDFWT, pKM1 and pKM2 were obtained in this study.

**DNA isolation, hybridization and sequencing.** Chromosomal DNA from *Synechocystis* 6803 cells was isolated as described by Grigorieva & Shestakov (1982). Southern hybridization and other routine DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). DNA sequencing was carried out by the dideoxynucleotide chain-termination procedure (Sanger *et al.*, 1977). The final sequence was determined from both strands of the DNA fragments.

**Transformation of *Synechocystis* 6803 cells.** For the transformation of WT cells of *Synechocystis* 6803 to herbicide resistance and for the creation of insertion mutants, we used a procedure adapted from the method described by Williams (1988). The recipient cells of *Synechocystis* 6803, grown for 4-5 d in liquid BG11 medium, were harvested, resuspended in fresh BG11 medium (10⁹ cells ml⁻¹), mixed with 0.5-2 μg transforming DNA and spread onto Millipore nitrocellulose filters (HA, 0.45 μm) resting on nonselective agar plates. After incubation of the plates for 18-20 h, the filters were transferred to plates containing selective agent (80 μg difunon ml⁻¹ or 10 μg kanamycin ml⁻¹). Transformants appeared within 7-10 d.

**Analysis of nucleotide and protein sequences.** This was performed using the Genetics Computer Group software package (Devereux *et al.*, 1984). For database searches the *BLAST* (Altschul *et al.*, 1990) network service was used. For a detailed comparison of the amino acid sequences, the *BESTFIT* program was used (Devereux *et al.*, 1984). Hydrophobicity of proteins was analysed by the method of Engelman *et al.* (1986) using the TopPred II program (von Heijne, 1992).

**Materials.** Enzymes for recombinant DNA work were from Fermentas; modified T7 polymerase (Sequenase) was from United States Biochemical; [α-³²P]dATP for radioactive labelling of DNA fragments and for DNA sequencing was from Radiopreparat; difunon was from Celamerck; diuron was from Serva; chlorpromazine, trifluoperazine and kanamycin were from Sigma.

**RESULTS AND DISCUSSION**

**Cross-resistance of DF1 and CPN6 mutants of *Synechocystis* 6803**

Herbicide-resistant mutant DF1 was selected on solid BG11 medium (Allen, 1968) containing 350 μM difunon (Shestakov & Bartsevich, 1993). This mutant was found to be cross-resistant to the calmodulin antagonists chlorpromazine and trifluoperazine. A number of spontaneous mutants, including the CPN6 mutant, were selected for resistance to 45 μM chlorpromazine. These mutants showed cross-resistance to difunon and trifluoperazine (Shestakov & Bartsevich, 1993). The DF1 and CPN6 mutants were subsequently found to be cross-resistant to the herbicide diuron. The resistance of the WT, DF1 and CPN6 strains to various inhibitors is shown in Table 1. The mutant strains show the highest degree of resistance to the herbicide difunon, which was chosen as a selective agent for the complementation experiments. DNA isolated from strains DF1 and CPN6 transformed the WT cells of *Synechocystis* 6803 to difunon resistance with high efficiency. All difunon-resistant transformants were also resistant to chlorpromazine, trifluoperazine and diuron. We suggest that mutants DF1 and CPN6 have mutations in one and the same gene.
Table 1. Resistance (in μM) of WT and mutant strains of *Synechocystis* 6803 to various growth inhibitors

The degree of resistance was determined by streaking cells on solid medium containing different concentrations of the inhibitors. The reported concentration is the threshold amount that allowed growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Difunon</th>
<th>Diuron</th>
<th>Chlorpromazine</th>
<th>Trifluoperazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>80</td>
<td>0.7</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>DF1</td>
<td>350</td>
<td>1.4</td>
<td>45</td>
<td>200</td>
</tr>
<tr>
<td>CPN6</td>
<td>350</td>
<td>1.4</td>
<td>45</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig. 1. (a) Restriction map of the 7.2 kb XbaI DNA fragment from the genome of the DF1 mutant cloned in the pDF1 plasmid. (b) Deletion analysis of the DF1 mutation. A number of restriction fragments from pDF1 were cloned in vector pUC19 and used to transform WT cells of *Synechocystis* 6803 to difunon resistance. Transformation and an absence of transformation to herbicide resistance are designated by + and −, respectively. The mutation was mapped on a 0.1 kb HindIII-PstI DNA segment. A, AsuII; B, BglII; E, EcoRI; H, HindIII; P, PstI; X, XbaI.

Fig. 2. Southern blot analysis of genomic DNA of *Synechocystis* 6803. Chromosomal DNA from WT *Synechocystis* 6803 cells was digested with BglII (lane 1), KpnI (lane 2), PstI (lane 3) and with XbaI (lane 4), fractionated on an agarose gel, transferred to a nitrocellulose filter and probed with a 32P-labelled 13 kb BglII-PstI DNA fragment from pPDF1.

WT cells of *Synechocystis* 6803 to difunon resistance. DNA fragments in the 6–9 kb range yielded the greatest number of transformants. These fragments were cloned into plasmid vector pUC19. Among 600 isolated clones, one recombinant plasmid (named pDF1) was found to transform WT cells of *Synechocystis* 6803 to difunon resistance. Fig. 1(a) shows a restriction map of the 7.2 kb XbaI DNA fragment cloned in pDF1.

To localize the mutation in DF1, a number of restriction fragments of the pDF1 insert were assayed for their ability to confer resistance to WT cells of *Synechocystis* 6803 (Fig. 1b). The mutation was mapped on a 0.1 kb HindIII-PstI DNA fragment. Southern blot analysis of the chromosomal DNA from WT cells indicated that the genetic information in this fragment is present as a single copy in the *Synechocystis* 6803 genome (Fig. 2).

The 1.3 kb BglII-PstI subfragment from the pDF1 insert was used as a probe to clone the homologous 7.2 kb XbaI DNA fragments from WT and CPN6 strains. Restriction maps of fragments cloned in plasmids pDFWT (WT strain) and pCPN6 (CPN6 strain) are identical to the map of the cyanobacterial DNA insert in the pDF1 plasmid. The deletion analysis showed that the mutation in CPN6 lay within a 1 kb BglII-EcoRI DNA fragment located near the DF1 mutation (data not shown).

The complete nucleotide sequence of the 3 kb HincII–AsuII region (Fig. 3b) from the 7.2 kb XbaI DNA fragment of pDFWT was determined. A large ORF, within which the DF1 and CPN6 mutations were mapped, was identified. This ORF was named *dspA* (coding drug sensory protein A; DspA). The *dspA* gene can encode a polypeptide of 663 amino acids with a predicted molecular mass of 74.5 kDa. A purine-rich region, which might serve as a potential ribosome-binding site, was found upstream of *dspA* (data not shown).

Cloning and sequencing the gene controlling resistance in mutants DF1 and CPN6

DNA extracted from DF1 mutant cells was digested with XbaI and fractionated by electrophoresis on a 0.6% low-melting-point agarose gel. Individual DNA fractions were cut from the gel and assayed for their ability to transform...
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Database analysis of the nucleotide sequence indicates that the N-terminal region of \textit{dspA} shares high similarity with the sequence of an unidentified ORF of 1095 bp which has been found 194 bp upstream of the \textit{psaD} gene (encoding subunit II of photosystem I) on the opposite strand in the \textit{Synechocystis} 6803 chromosome (Reilly \textit{et al.}, 1988). This ORF starts from the same initiation codon as \textit{dspA}, but terminates before the \textit{dspA} termination codon due to some difference in nucleotide sequences (data not shown). The sequenced \textit{HincII–AsuI} DNA fragment reported here also contains the N-terminal part of the \textit{psaD} gene (Fig. 3), the nucleotide sequence of which (data not shown) is identical to that determined previously (Reilly \textit{et al.}, 1988). In the \textit{Synechocystis} 6803 genome there is only one copy of \textit{psaD} (Reilly \textit{et al.}, 1988). Therefore, we have cloned and analysed the same region of the \textit{Synechocystis} 6803 chromosome. We suggest that the 1095 bp ORF determined by Reilly and collaborators is only a part of the \textit{dspA} gene.

**Mutational analysis of the \textit{dspA} gene**

To determine the molecular nature of the spontaneous mutations in strains DF1 and CPN6, the corresponding 0.24 kb \textit{EcoRI–PstI} DNA fragment from pDF1 and 1.1 kb \textit{BglII–EcoRI} fragment from pCPN6 were sequenced. A comparison of nucleotide sequences from strains DF1 and WT revealed a deletion of three nucleotides in the DF1 mutant leading to the loss of glutamine at position 521 (Fig. 3c). A large duplication of 66 bp was found in the nucleotide sequence of \textit{dspA} from the mutant strain CPN6 (Fig. 3c).

To confirm the involvement of \textit{dspA} in control of cross-resistance to different inhibitors, we created targeted insertion mutant strains. A Km\textsuperscript{R} gene cartridge from the plasmid \textit{pUC4K} was inserted at \textit{BglII} or \textit{SnaBI} sites located in different parts of \textit{dspA} (Fig. 3a). To create such mutants, plasmids \textit{pKM1} and \textit{pKM2} were constructed as follows (Fig. 4): 1.1 kb \textit{HincII} and 2 kb \textit{BglII–AsuI} fragments from pDFWT were respectively cloned into \textit{HincII–AsuI} DNA and complete segregation of homozygous mutants were confirmed by Southern hybridization analysis (data not shown). Km\textsuperscript{R} transformants were checked for resistance to difunon and chlorpromazine. All assayed transformants were resistant to difunon as well as to chlorpromazine. These data indicate that the functional activity of the \textit{dspA} gene is not essential for growth of \textit{Synechocystis} 6803, although inactivation of this gene did result in cross-resistance to some inhibitors.

**Analysis of the DspA protein amino acid sequence**

Database searches of the deduced amino acid sequence of the DspA protein indicated that the C-terminal region of DspA exhibits similarity with HPKs. DspA shares the highest similarity with the C-terminus of the Bacillus

![Fig. 4. Schematic illustration of the pKM1 and pKM2 plasmids constructed for cartridge mutagenesis of the \textit{dspA} gene. Kmr gene cartridge; cyanobacterial DNA; pUC19 vector. A, AsuI; B, BglII; Bm, BamHI; Hc, Hincll; S, SnaBI; Sm, SmaI.](image)
Fig. 5. Comparison of the deduced amino acid sequences of C-terminal portions of \textit{Synechocystis} 6803 DspA and \textit{B. subtilis} PhoR proteins (Seki \textit{et al.}, 1988). Vertical lines connect identical amino acids; similar amino acids are indicated by dots (· and :) an asterisk above the sequence identifies conserved residues for the HPK family; #, putative site of autophosphorylation.

**Fig. 6.** Hydropathy profile of DspA. Analysis was made by the method of Engelman \textit{et al.} (1986). The size of the averaging window was 11 amino acid residues.

### Concluding remarks

Cyanobacteria provide an experimental system for cloning new genes via complementation of characterized mutations. Using such an approach, we have discovered a new gene (\textit{dspA}) of the cyanobacterium \textit{Synechocystis} 6803. Mutations in this gene result in cross-resistance to some herbicides and calmodulin antagonists. These inhibitors have various chemical structures and act on different targets in the cell. Computer analysis shows that the deduced amino acid sequence of the DspA protein exhibits similarity with HPKs and the N-terminus of DspA contains two putative transmembrane segments. We speculate that the DspA protein could function as a sensory HPK and participate in sensing of chemicals. The loss of such functions could alter the cells' permeability or metabolism in a way that affects degradation of inhibitors. However, the exact mechanism of resistance in \textit{dspA} mutants and the primary physiological function of DspA are not clear at present.

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### REFERENCES


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