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# Transformation of *Synechococcus* with a gene for choline oxidase enhances tolerance to salt stress

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## Abstract

Choline oxidase, isolated from the soil bacterium *Arthrobacter globiformis*, converts choline to glycine-betaine (N-trimethylglycine) without a requirement for any cofactors. The gene for this enzyme, designated *codA*, was cloned and introduced into the cyanobacterium *Synechococcus* sp. PCC 7942. The *codA* gene was expressed under the control of a strong constitutive promoter, and the transformed cells accumulated glycinebetaine at intracellular levels of 60–80 mM. Consequently the cells acquired tolerance to salt stress, as evaluated in terms of growth, accumulation of chlorophyll and photosynthetic activity.

## Introduction

Glycinebetaine (called betaine hereinafter) is accumulated in the cells of a number of halotolerant plants and bacteria as an adaptive response to saline and/or water-deficient environments [11, 16, 26]. Betaine acts as an osmoregulatory substance, maintaining the osmotic balance with the environment [27] and it also prevents the dissociation of soluble enzymes at high concentrations of NaCl [7, 8]. Betaine protects the photosystem II complex of the thylakoid membranes against the high-salt-induced dissociation of the extrinsic proteins and the manganese cluster [17, 19, 20, 23].

In both *Escherichia coli* and *Spinacia oleracea* betaine is synthesized by the two-step oxidation of choline via betainealdehyde as an intermediate. In *E. coli*, the oxidation reactions are catalyzed by two dehydrogenases: a membrane-bound oxygen-dependent choline dehydrogenase [14] and a soluble NAD-dependent betainealdehyde dehydrogenase [6]. In *S. oleracea*, a ferredoxin-dependent monooxygenase is involved in the first

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X84895.

step [3] and NAD-dependent betainealdehyde dehydrogenase is responsible for the second step [33].

By contrast, choline oxidase from the soil bacterium *Arthrobacter globiformis* oxidizes choline to betaine without a requirement for any cofactors [9, 10]. The gene for choline oxidase was first isolated from *A. pascens* and it was introduced into *E. coli* mutant that was defective in betaine synthesis. The expression of this gene improved the salt tolerance of the mutant [28]. However, the nucleotide sequence of the gene for choline oxidase from *A. pascens* has not been reported.

In the present study, we isolated the *codA* gene for choline oxidase from *A. globiformis* and introduced it into the cyanobacterium *Synechococcus* sp. PCC 7942. The transformed cells accumulated a high level of betaine and acquired the ability to grow under high-salt conditions.

## Materials and methods

### *Organisms and culture conditions*

*Arthrobacter globiformis* was generously provided by Asahi Chemical Industrial Company (Shizuoka, Japan). The cells were grown at 30 °C for 20 h in the medium recommended by Ikuta *et al.* [9].

*Synechococcus* sp. PCC 7942 and its transformants were grown at 30 °C in BG11 medium [30] under illumination from incandescent lamps at 70 $\mu E\ m^{-2}\ s^{-1}$ with aeration by air that contained 1% $CO_2$. For the evaluation of tolerance to high-salt conditions, cells were grown in BG11 medium that contained NaCl at various concentrations plus 30 μg/ml spectinomycin. Choline chloride (Katayama Chemical, Osaka, Japan) was added to the culture medium to a final concentration of 1 mM as the precursor for the synthesis of betaine.

### *Determination of partial amino acid sequences of choline oxidase*

Choline oxidase from *A. globiformis* (purchased from Sigma Chemical Co., St. Louis, MO) was cleaved into peptide fragments by incubation with cyanogen bromide in 70% formic acid for 14 h [25]. Resultant fragments were separated by SDS-PAGE and then electroblotted onto a polyvinylidine difluoride membrane (AE6660; Atto, Tokyo, Japan). The amino-terminal amino acid sequences of choline oxidase and its fragments were determined with an automatic protein sequence analyzer (model 477A; Applied Biosystems, Foster City, CA).

### *Construction of the genomic DNA library*

Genomic DNA was isolated from cells of *A. globiformis* as follows. The cells were disrupted with a French press (40K; SLM Instruments, Urbana, IL) at 160 MPa in a buffer that contained 10 mM Tris-HCl pH 8.0, 100 mM NaCl, and 1 mM EDTA. The DNA was isolated from the homogenate by treatment with phenol and subsequent centrifugation on a gradient of CsCl-ethidium bromide [29].

The isolated DNA was used to construct a genomic library of *A. globiformis* in the *λDASH*II vector according to the protocol that was supplied with the cloning kit (Lambda *DASH*II/*Bam* HI cloning kit; Stratagene, La Jolla, CA). The phage library was packaged using a standard kit (Gigapack II gold packaging kit; Stratagene).

### *Cloning of the* codA *gene of* A. globiformis

On the basis of the amino-terminal sequence of choline oxidase, we selected two sets of amino acid residues, residues 1 to 5 and 15 to 19 (counted from the amino terminus). Referring to these sequences, we synthesized two oligonucleotides, namely, 5′-ATGCA(CT)AT(ACT)-GA(CT)AA-3′ and 5′-AC(ACGT)AC(AGT)-AT(AG)TA(AG)TC-3′ for use as forward and reverse primers, respectively, for the amplification of part of the *codA* gene by PCR with the genomic DNA of *A. globiformis* as template. The amplification was performed by 30 cycles of 1 min at 94 °C for denaturation, 3.5 min at 32 °C for

annealing, and 2.5 min at 72 °C for synthesis. The amplified products were subcloned into the T-A cloning vector (TA Cloning kit; Invitrogen, San Diego, CA). Nucleotide sequences of the inserts of two clones were determined with an automatic DNA sequencer (model 373A; Applied Biosystems) and we found that both included partial sequences of the *codA* gene.

The insert of one of the plasmids was used as a probe to screen the genomic DNA library of *A. globiformis* by the standard method [29]. $\lambda$DNAs of hybridized phage clones were isolated and digested with the restriction enzymes *Xba* I and *Xho* I. The resultant fragments were subjected to Southern blot analysis. The DNA fragment that hybridized with the probe was isolated and subcloned into plasmid pBluescript (SK + ) (Stratagene). The insert in pBluescript was mapped with the restriction enzymes *Bst* EII, *Pst* I, *Sac* I, *Sal* I, and *Sma* I. The fragments from digestions with *Pst* I and *Sal* I, respectively, were separately subcloned into pBluescript and their nucleotide sequences were determined with the automatic DNA sequencer.

### *Transformation of* Synechococcus *sp. PCC 7942*

The plasmid pBluescript that carried the *codA* gene (Fig. 1A) was digested with *Bst* EII and *Sma* I. The *Bst* EII-cohesive end was filled in by the Klenow fragment of DNA polymerase (Takara, Tokyo, Japan). The blunt-ended fragment, which contained the coding region of the *codA* gene and a putative ribosome-binding site, was inserted into the *Sma* I site of plasmid pAM1044 (provided by Dr Susan S. Golden, Texas A&M University). This plasmid carried the conII promoter, a spectinomycin-resistance cartridge, and intergenic regions of the chromosomal DNA of *Synechococcus* sp. PCC 7942 that allowed the integration of the plasmid into the chromosome by homologous recombination (Susan S. Golden, personal communication). The orientation of the gene in the construct was confirmed by restriction analysis with *Sac* I.

Cells of *Synechococcus* sp. PCC 7942 were transformed with plasmids pAM1044 and pAM1044/*codA* as described by Elhai *et al.* [5]. Transformed cells were selected on agar plates that contained spectinomycin at 30 $\mu$g/ml in BG11 medium. After several transfers of single colonies to agar plates that contained spectinomycin, we confirmed by PCR that all copies of the native chromosome had been replaced by a chromosome that carried the spectinomycin-resistance cartridge and the *codA* gene.

### *Western blot analysis*

For western blot analysis, total proteins extracted from the transformed cells were subjected to SDS-PAGE. After electrophoresis, the proteins were electroblotted on a nitrocellulose membrane (BA85; Schleicher & Schuell, Dassel, Germany). Immunological detection was performed according to the protocol supplied with a Vecstain ABC-PO (rabbit IgG) kit (Vector laboratories, Burlingame, CA) using polyclonal antiserum raised in rabbit against the choline oxidase from *A. globiformis* that had been purchased from Sigma Chemical Co.

### *Quantitation of betaine*

Transformed cells were grown in one liter of BG11 medium supplemented with 1 mM choline chloride in the presence of NaCl at various concentrations plus 30 $\mu$g/ml spectinomycin. The cells were harvested and treated with 1 M $H_2SO_4$ at 25 °C for 20 h, and then the cell debris was removed by centrifugation at 3000 × ***g*** for 15 min. The extracted betaine was recovered from the supernatant by the periodide precipitation method [31]. The resultant betaine periodide was collected by centrifugation at 3000 × ***g*** and was dissolved in 1 ml of methanol-$d_4$ (Wako Pure Chemical Industries, Osaka, Japan) that contained 2 mM 2-methyl-2-propanol (Wako Pure Chemical Industries) as an internal standard. This solution was transferred to an NMR tube, and the $^1$H NMR spectrum was recorded at 25 °C

with an NMR spectrometer (AMX 360 Wb; Bruker, Karlsruhe, Germany) with a pulse time of 5.0 μs and an acquisition time of ca. 4 s. Betaine was quantified from comparisons of integrated peak intensities. The concentration of betaine in a cell was calculated from the number of cells in a unit volume and the intracellular volume, which was estimated from electron micrographs of cell morphology.

*Evaluation of tolerance to salt stress*

The tolerance to salt stress of the transformed cells was evaluated in terms of growth, the accumulation of chlorophyll and photosynthetic activity. Cells were cultivated first at 30 °C for 3 days in BG11 medium supplemented with 1 mM choline chloride and 30 μg/ml spectinomycin. Then the cells were transferred to BG11 medium that contained 0.4 M NaCl, 1 mM choline chloride and 30 μg/ml spectinomycin. Growth was monitored in terms of optical density at 730 nm. The accumulation of chlorophyll was determined by the method of Arnon *et al.* [1]. Photosynthetic activity was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode. Red actinic light at 2 mE $m^{-2}$ $s^{-1}$ was provided from an incandescent lamp after passage through a heat-absorbing optical filter (HA50; Hoya, Tokyo, Japan) and a red optical filter (R-60; Toshiba, Tokyo, Japan).

## Results

*Cloning of the* codA *gene from* Arthrobacter globiformis

The amino-terminal sequence of 21 amino acid residues of choline oxidase was determined (see Fig. 2, the first underlined sequence). Two degen-

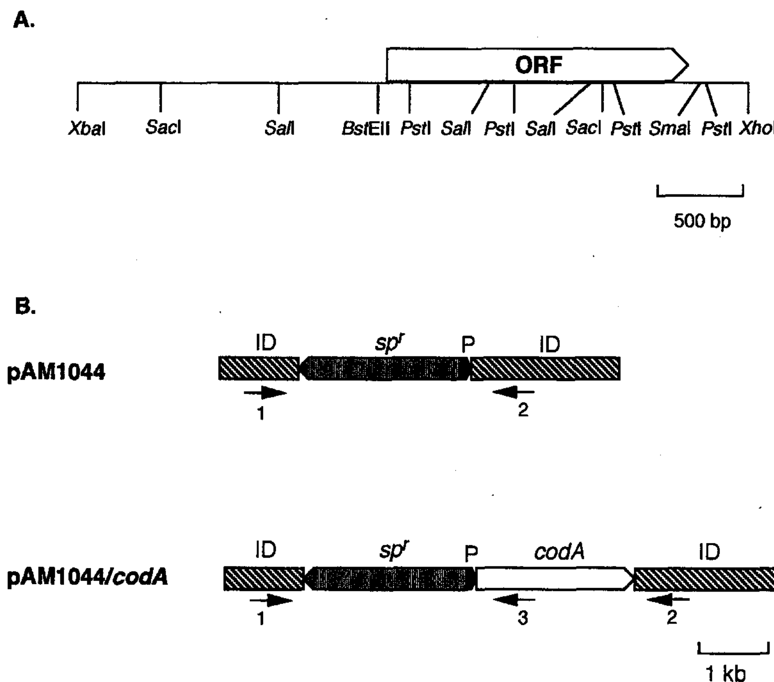


*Fig. 1.* A. A restriction map of the 3.6 kb *Xba* I-*Xho* I insert that contained the *codA* gene. The nucleotide sequence was determined in the region from the first *Sal* I site to *Xho* I site. B. Schematic representation of the pAM1044/*codA* construct. ID, intergenic chromosomal DNA of *Synechococcus* sp. PCC 7942; P, conII promoter; *spʳ*, spectinomycin-resistance gene cartridge. Arrows indicate the primers used for PCR.

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-360 GGGAATATCCGTCGTCGTAGACGAGCCCTTCGGCCCGTGTAAAGGTGGAGACCTTCCACACCGAGGACGAGGCCGTCGCGACCGCCAACG
-270 ACACCAACTACGGGCTGTCCGGCGCGGTCCTGGACCCAGGACGCCGGCAAGACGCAGCGCGTGGCCGGCCGGCTGCGACACGGCACCGTC
-180 TGGATCAACGACTTCCACCCCTACCTCCCACAGACCGAGTGGGGCGGCTTCGGCCAGTCCGGCGTCGGCCGCGAACTCGGCCCGACCGGC
 -90 CTGGCCGAGTACCAGGAGGCCAAGCACATCTACCAGAACACCAGCCCGCAGGTCACCGGCTGGTTCGCTGACCACGGCAAGGAGAACTAG

   1 ATGCACATCGACAACATCGAGAACCTGAGCGACAGGGAGTTCGACTACATCGTCGTCGGCGGCGGGTCCGCCGGGGCCGCCGTCGCCGCC
   1  M  H  I  D  N  I  E  N  L  S  D  R  E  F  D  Y  I  V  V  G  G  G  S  A  G  A  A  V  A  A

  91 CGGCTGAGCGAGGATCCCGCAGTGAGCGTGGCGCTGGTGGAGGCCGGCCCGGATGACCGCGGCGTGCCCGAGGTGCTGCAGCTGGACCGC
  31  R  L  S  E  D  P  A  V  S  V  A  L  V  E  A  G  P  D  D  R  G  V  P  E  V  L  Q  L  D  R

 181 TGGATGGAGCTGCTGGAATCGGGCTACGACTGGGACTACCCGATCGAGCCGCAGGAGAACGGCAACTCCTTCATGCGCCATGCCCGTGCC
  61  W  M  E  L  L  E  S  G  Y  D  W  D  Y  P  I  E  P  Q  E  N  G  N  S  F  M  R  H  A  R  A

 271 AAGGTCATGGGCGGCTGCTCCAGCCACAACTCCTGCATCGCCTTCTGGGCCCCGCGCGAGGACCTGGACGAGTGGGAGGCCAAGTACGGC
  91  K  V  M  G  G  C  S  S  H  N  S  C  I  A  F  W  A  P  R  E  D  L  D  E  W  E  A  K  Y  G

 361 GCCACCGGCTGGAACGCCGAGGCGGCCTGGCCGCTGTACAAGCGGCTGGAAACCAACGAGGACGCGGGCCCGGACGCGCCGCACCACGGG
 121  A  T  G  W  N  A  E  A  A  W  P  L  Y  K  R  L  E  T  N  E  D  A  G  P  D  A  P  H  H  G

 451 GACTCCGGCCCCGTGCACCTGATGAACGTGCCCCCGAAGGACCCGACCGGCGTCGCGCTCCTGGACGCCTGCGAGCAGGCCGGCATCCCG
 151  D  S  G  P  V  H  L  M  N  V  P  P  K  D  P  T  G  V  A  L  L  D  A  C  E  Q  A  G  I  P

 541 CGCGCGAAGTTCAACACCGGCACCACCGTGGTCAACGGCGCCAACTTCTTCCAGATCAACCGGCGCGCGGACGGCACCCGCTCCTCCAGC
 181  R  A  K  F  N  T  G  T  T  V  V  N  G  A  N  F  F  Q  I  N  R  R  A  D  G  T  R  S  S  S

 631 TCGGTCTCCTACATCCACCCGATCGTCGAGCAGGAGAACTTCACCCTGCTAACCGGCCTGCGCGCCCGCCAGCTGGTGTTCGACGCGGAC
 211  S  V  S  Y  I  H  P  I  V  E  Q  E  N  F  T  L  L  T  G  L  R  A  R  Q  L  V  F  D  A  D

 721 AGGCGCTGCACCGGCGTCGACATCGTGGACTCCGCCTTCGGCCGCACCCATCGGCTGACGGCGCGCAATGAAGTCGTGCTCTCCACCGGC
 241  R  R  C  T  G  V  D  I  V  D  S  A  F  G  R  T  H  R  L  T  A  R  N  E  V  V  L  S  T  G

 811 GCGATCGATACGCCGAAGCTGTTGATGCTCTCCGGAATCGGCCCCGCCGCCCACCTCGCCGAGCACGGCATCGAGGTCCTTGGTGGACTC
 271  A  I  D  T  P  K  L  L  M  L  S  G  I  G  P  A  A  H  L  A  E  H  G  I  E  V  L  G  G  L

 901 CCCCGGCGTGGGCGAGCACCTGCAGGACCACCCGGAAGGCGTGGTGCAGTTCGAGGCCAAGCAGCCCATGGTCGCCGAGTCCACGCAGTG
 301  P  R  R  G  R  A  P  A  G  P  P  G  R  R  G  A  V  R  G  Q  A  A  H  G  R  R  V  H  A  V

 991 GTGGGAGATCGGCATCTTCACCCCCACCGAGGACGGCCTGGACCGCCCCGACCTGATGATGCACTACGGCTCCGTGCCGTTCGACATGAA
 331  V  G  D  R  H  L  H  P  H  R  G  R  P  G  P  P  R  P  D  D  A  L  R  L  R  A  V  R  H  E

1081 CACCCTGCGGCACGGCTACCCCACCACGGAGAACGGGCTTCAGCCTCACCCCGAACGTCACGCACGCCCGCTCCCGCGGCACTGTCCGGC
 361  H  P  A  A  R  L  P  H  H  G  E  R  A  S  A  S  P  R  T  S  R  T  P  A  P  A  A  L  S  G

1171 TGCGCAGCCGCGACTTCCGCGATAAGCCCATGGTCGACCCGCGCTACTTCACCGACCCAGAAGGGCCATGACATGCGCGTCATGGTCGCC
 391  C  A  A  A  T  S  A  I  S  P  W  S  T  R  A  T  S  P  T  Q  K  G  H  D  M  R  V  M  V  A

1261 GGCATCCGCAAGGCCCGCGAAATCGCCGCCCAGCCCGCCATGGCGGAATGGACCGGCCGCGAGCTCTCCCCCGGCGTCGAGGCGCAGACC
 421  G  I  R  K  A  R  E  I  A  A  Q  P  A  M  A  E  W  T  G  R  E  L  S  P  G  V  E  A  Q  T

1351 GACGAGGAGCTGCAGGACTACATCCGCAAGACGCACAACACCGTCTACCACCCCGTGGGCACCGTGCGCATGGGCGCGGTCGAGGACGAG
 451  D  E  E  L  Q  D  Y  I  R  K  T  H  N  T  V  Y  H  P  V  G  T  V  R  M  G  A  V  E  D  E

1441 ATGTCCCCGCTCGACCCCGAGCTGCGGGTCAAGGGCGTCACCGGTCTGCGCGTCGGCGACGCCTCGGTCATGCCCGAGCACGTGACCGTC
 481  M  S  P  L  D  P  E  L  R  V  K  G  V  T  G  L  R  V  G  D  A  S  V  M  P  E  H  V  T  V

1531 AACCCCAACATCACCGTCATGATGATCGGCGAGCGCTGCGCGGACCTTATCCGCTCCGCCCGCGCCGGTGAAACAACGACGGCGGACGCC
 511  N  P  N  I  T  V  M  M  I  G  E  R  C  A  D  L  I  R  S  A  R  A  G  E  T  T  T  A  D  A

1621 GAGCTGAGCGCGGCCCTCGCCTAAGCGGGAGCGGCCAGCCGCGGGGCCTGTCCGGAACCACCTGGCGGGCCCCGCATGGGGCCGGACACA
 541  E  L  S  A  A  L  A  *

1711 ATGCCGGTAACTAAGGGTGCGGAAGCAGTCCTGCTTCCACACCCGCGTTTTGCACGCCCGGGCCGGCAACTGGCCCGGCCGGCTAAGCCG
1801 AAGGTCTTCCGGGGGCGGGCCGGATCGCTGCGGGCAGTCCGTCGGCCAGCCGCTGCAGCGTGCCGGCGGTAATGGCGGTGTAGGCAGGGA
1891 TCGCGTCGGGGTAGATGTACTCGTTGCGGGCGTGCGCGCCGTCGCCCACCGCGCCCAGGCCGCACAGGACCGGGATGCCGAGGGCGGAGA
1981 CGAAGTTGGCGTCGCTGCCCCCGCCCACCGAGGCGGTTTCCAGCTCCCGGCCCTGCTCCA
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*Fig. 2.* The nucleotide sequence of the *codA* gene for choline oxidase of *Arthrobacter globiformis* and the deduced amino acid sequence. The underlining indicates the amino acid sequences that were directly determined by Edman degradation. The shaded amino acid sequence indicates the putative FAD-binding site. The double underlining indicates a putative ribosome-binding site.

erate primers were synthesized on the basis of this sequence and were used for amplification of part of the *codA* gene by PCR with the genomic DNA of *A. globiformis* as template. Amplified products of about 50 bp were subcloned into the T-A cloning vector, and the nucleotide sequences of the inserts of two clones were determined. Both clones contained a nucleotide sequence that corresponded to the amino-terminal sequence of choline oxidase. One of the clones was arbitrarily selected for further experiments.

A genomic DNA library prepared from *A. globiformis* was screened with the insert of the selected plasmid. Four hybridizing phage clones were obtained from about 3000 recombinants. $\lambda$DNAs of the phages were isolated and subjected to Southern blot analysis. All four $\lambda$DNAs yielded a *Xba* I-*Xho* I fragment of 3.6 kb that hybridized with the probe. This fragment was subcloned into pBluescript. Figure 1A shows a map of the restriction sites of the insert.

The nucleotide sequence of the region (about 2.5 kb) from the first *Sal* I site to the *Xho* I site was determined, and an open-reading frame was found. Figure 2 shows the nucleotide sequence of 1641 bp and the amino acid sequence deduced from the open-reading frame, which encodes a polypeptide of 547 amino acid residues. The experimentally determined amino-terminal and internal amino acid sequences of choline oxidase (underlined in Fig. 2) matched the deduced amino acid sequence. The amino acid motif corresponding to a putative FAD-binding site (the shaded region in Fig. 2) was found in the sequence of choline oxidase [32].

### *Insertion of the* codA *gene into the chromosome of* Synechococcus *sp. PCC 7942*

The plasmid pAM1044/*codA* was introduced into *Synechococcus* sp. PCC 7942, and the resultant transformant strain was designated PAMCOD. *Synechococcus* sp. PCC 7942 that had been transformed with pAM1044 alone, designated strain PAM, was also produced for use as a control. Analysis by PCR indicated that all the copies of the native chromosome had been completely replaced by recombinant chromosomes (Fig. 3). When the DNA from strain PAM was used as template with primers 1 and 2 (Fig. 1B), only one band appeared, corresponding to a fragment of 2.4 kb (Fig. 3B, lane c), suggesting that pAM1044 had been inserted into the chromosomes. The absence of a 400 bp band, which would correspond to the product of PCR from the chromosome of the wild-type strain (Fig. 3, lane b), confirmed the complete replacement of native chromosomes by recombinant chromosomes in strain PAM. When an attempt was made to amplify the DNA from PAMCOD with primers 1 and 2, no bands corresponding to either wild-type chromosomes or recombinant chromosomes were observed. It is likely that the high G-C content of the *codA* sequence impeded amplification by PCR. However, when primers 1 and 3 (Fig. 1B) were used, a band of 2.6 kb was amplified (Fig. 3, lane d), which corresponded to the calculated size

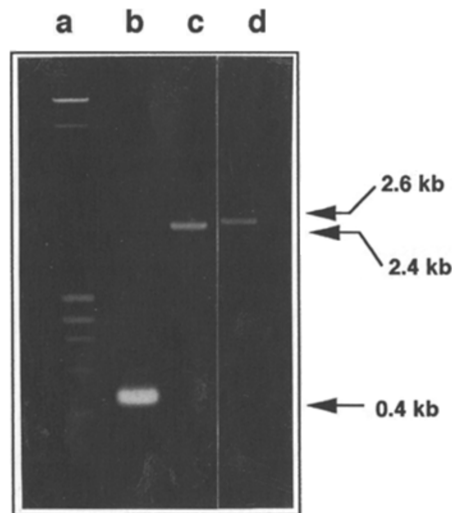


*Fig. 3.* Analysis by PCR of the inserted recombinant DNA in the chromosomes of the PAM and PAMCOD strains of *Synechococcus*. Lane a, $\lambda$-*Hind* III/øx 174-*Hae* III fragments (molecular size markers); lane b, the product of PCR obtained with primers 1 and 2 and the DNA from wild-type cells of *Synechococcus* sp. PCC 7942 as template; lane c, the product of PCR with primers 1 and 2 and the DNA from cells of strain PAM as template; lane d, the product of PCR with primers 1 and 3 and the DNA from cells of strain PAMCOD as template.

of the amplified product. These findings indicated that the *codA* gene and the spectinomycin-resistance cartridge had been inserted together into the chromosome of strain PAMCOD.

### *Expression of the* codA *gene and the accumulation of betaine in* Synechococcus *strain PAMCOD*

The expression of the *codA* gene in strain PAMCOD was examined by western blot analysis (Fig. 4). Only one band was detected in the extract from strain PAMCOD that corresponded to a protein with a molecular mass of 60 kDa, namely, the molecular mass of choline oxidase. This result indicated that the *codA* gene had been expressed under the control of the conII promoter in *Synechococcus* sp. PCC 7942.

The concentration of betaine in cells of strains PAM and PAMCOD was determined from the content of betaine, the number of cells per unit volume and the average volume of the cytoplasm of a cell. The shape of cells, determined by negative staining, was approximated by a cylinder of 0.96 $\mu$m in diameter and 2.49 $\mu$m in length. The thickness of the cell wall determined by a thin-section method was 0.07 $\mu$m. Therefore, the cytoplasm of a single cell was determined to be equivalent to a cylinder of 0.82 $\mu$m in diameter and 2.35 $\mu$m in length. The volume of the cytoplasm of a single cell was calculated to be 1.24 $\mu m^3$. Table 1 shows the concentrations of betaine in cells, as estimated in this way. Betaine was not detected in strain PAM. The concentration of betaine in cells of strain PAMCOD ranged from 60 to 80 mM at concentrations of NaCl from zero to 0.4 M.

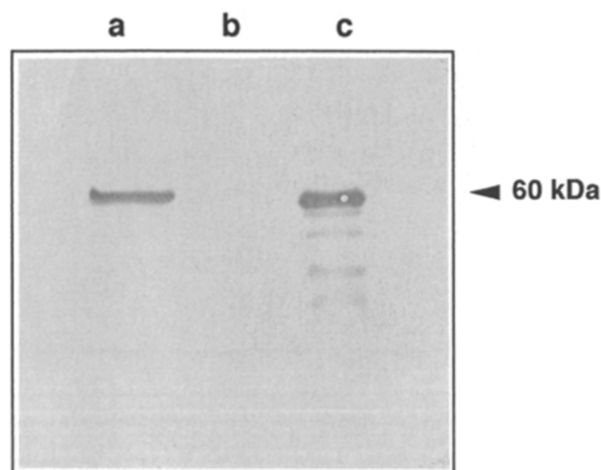


*Fig. 4.* Western blot analysis of the expression of choline oxidase in the PAM and PAMCOD strains of *Synechococcus*. Lane a, 5 $\mu$g protein extracted from strain PAMCOD; lane b, 5 $\mu$g protein extracted from strain PAM; lane c, 25 ng of choline oxidase of *Arthrobacter globiformis* purchased from Sigma Chemical Co.

*Table 1.* Concentrations of betaine in cells of *Synechococcus* strains PAM and PAMCOD. Cells were grown at 30 °C in BG11 medium supplemented with 1 mM choline chloride and designated concentrations of NaCl. The results were obtained from two independent experiments.

| NaCl concn., M | Betaine (mM) | |
|---|---|---|
| | PAM | PAMCOD |
| 0 | 0 | $60 \pm 4$ |
| 0.2 | 0 | $65 \pm 6$ |
| 0.3 | 0 | $77 \pm 3$ |
| 0.4 | 0 | $77 \pm 4$ |

### *Tolerance of* Synechococcus *strain PAMCOD to salt stress*

Cells of *Synechococcus* strains PAM and PAMCOD were grown in BG11 medium supplemented with 0.4 M NaCl and 1 mM choline chloride and their ability to tolerate salt stress was examined by monitoring growth, the accumulation of chlorophyll, and photosynthetic activity.

Figure 5 shows the growth of the cells of strains PAM and PAMCOD in the presence of 0.4 M NaCl and in its absence. Whereas cells of both strains grew rapidly, and at about the same rate, in the absence of NaCl, the presence of 0.4 M NaCl remarkably retarded the growth of strain PAMCOD and completely prevented the growth of strain PAM. Elimination of choline chloride from BG11 medium did not affect the growth profile of strain PAM either in the presence or absence of 0.4 M NaCl. Nonetheless, the results

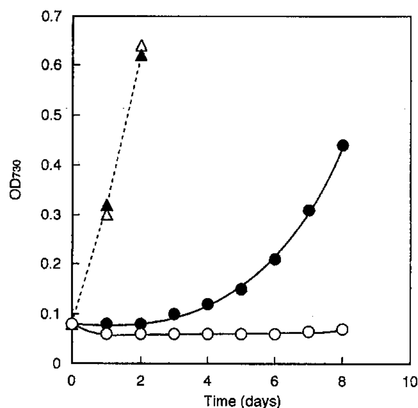


*Fig. 5.* Growth of cells of strains PAM and PAMCOD of *Synechococcus* in the presence of 0.4 M NaCl. Cells were first cultivated at 30 °C for 3 days in the light at 70 $\mu$E $m^{-2}$ $s^{-1}$ in BG11 medium supplemented with 1 mM choline chloride plus 30 $\mu$g/ml spectinomycin. Then the cells were transferred at time zero to BG11 medium supplemented with 0.4 M NaCl, 1 mM choline chloride and 30 $\mu$g/ml spectinomycin, and growth was allowed to continue under the same conditions. In the control experiment, the cells were grown in the absence of NaCl. ○, Strain PAM in 0.4 M NaCl; ●, strain PAMCOD in 0.4 M NaCl; Δ, strain PAM in the absence of NaCl; ▲, strain PAMCOD in the absence of NaCl.

clearly demonstrated that strain PAMCOD was much more tolerant than strain PAM to the salt stress.

Figure 6 shows the changes in chlorophyll content of cells of strains PAM and PAMCOD dur-

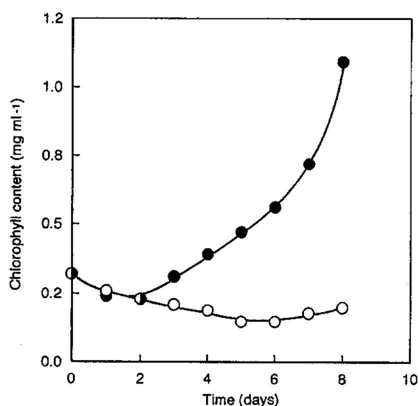


*Fig. 6.* Changes in chlorophyll content during growth in the presence of 0.4 M NaCl. Conditions for the initial culture and subsequent growth were the same as those described in the legend to Fig. 5. ○, strain PAM; ●, strain PAMCOD.

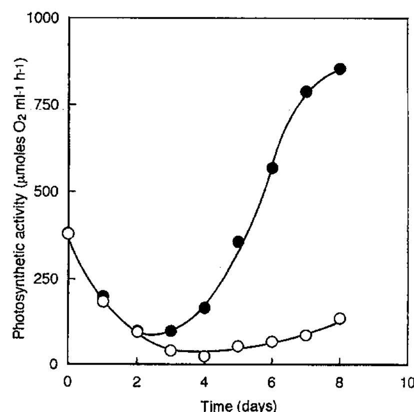


*Fig. 7.* Changes in photosynthetic activity during growth in the presence of 0.4 M NaCl. Conditions for the initial culture and subsequent growth were the same as those described in the legend to Fig. 5. ○, strain PAM; ●, strain PAMCOD.

ing incubation in 0.4 M NaCl. The chlorophyll content declined slowly in cells of strain PAM. By contrast, it increased in cells of strain PAMCOD after a lag period. This result suggests that strain PAMCOD synthesized and accumulated chlorophyll in 0.4 M NaCl.

Figure 7 shows the changes in the photosynthetic activity of strains PAM and PAMCOD during incubation in 0.4 M NaCl. In both types of cell, the photosynthetic activity decreased during the initial period of incubation in 0.4 M NaCl. However, subsequently, the cells of strain PAMCOD slowly recovered their photosynthetic activity. The results in Fig. 7 also clearly demonstrate that the cells of strain PAMCOD had acquired tolerance to salt stress.

## Discussion

In this study, we isolated the *codA* gene for choline oxidase from *A. globiformis*. Since choline oxidase is a flavoprotein [22], we compared its deduced amino acid sequence with those of other flavoproteins, such as methanol oxidase of *Hansenula polymorpha* [15], choline dehydrogenase and succinate dehydrogenase of *E. coli* [13, 34]. Figure 8 shows that a conserved sequence, GXGXXG, and an array of amino acids typical of an FAD-binding site [32], both of which are

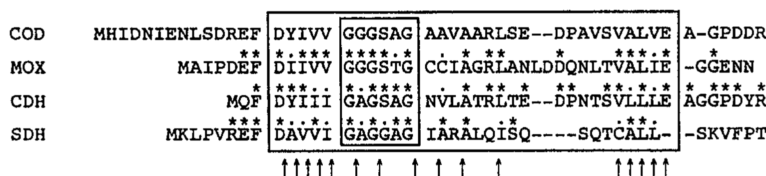


*Fig. 8.* A comparison of the amino-terminal sequence of choline oxidase with those of various flavoproteins. COD, choline oxidase of *Arthrobacter globiformis*; MOX, methanol oxidase of *Hansenula polymorpha* [15]; CDH, choline dehydrogenase of *Escherichia coli* [13]; SDH, succinate dehydrogenase of *E. coli* [34]. Identical amino acids in choline oxidase and other flavoproteins are indicated by asterisks. Arrows indicate the array of amino acids that is typical of an FAD-binding site [32]. The highly conserved amino acid sequence and the putative FAD-binding site are enclosed by small and large rectangles, respectively.

characteristic of flavoproteins, were found in the amino-terminal region of the product of the *codA* gene.

The *codA* gene was successfully inserted into the chromosome of *Synechococcus* sp. PCC 7942. Western blot analysis indicated that the *codA* gene was expressed under the control of the conII promoter, which is a constitutive promoter in *E. coli.* Cells of *Synechococcus* strain PAMCOD transformed with the *codA* gene accumulated betaine up to 80 mM when they were grown in medium supplemented with 1 mM choline chloride. This result suggests that *Synechococcus* sp. PCC 7942 is able to take up exogenously supplied choline and that choline is converted inside cells to betaine by choline oxidase. In several bacteria, the transport of choline is induced by salt stress, resulting in accumulation of a large amount of betaine [2, 12]. However, we found that the concentration of NaCl in the medium did not significantly affect the level of accumulation of betaine in cells of strain PAMCOD. It seems likely that the transporter responsible for the uptake of choline is constitutively operative in *Synechococcus* sp. PCC 7942.

When choline oxidase catalyzes the oxidation of choline to betaine by molecular oxygen, $H_2O_2$ is produced simultaneously [9, 10]. $H_2O_2$ is a hazardous compound in the cell. Scavengers of $H_2O_2$, such as catalase and ascorbate peroxidase, are present at high levels in *Synechococcus* sp. PCC 7942 [18] and must immediately degrade $H_2O_2$ when it is synthesized. The details of the regulation of expression of these enzymes by $H_2O_2$ have not been determined.

The cells of halophilic bacteria accumulate betaine to maintain an osmotic balance with their environment [4]. Higher plants, such as spinach, accumulate betaine, in particular in their chloroplasts, in response to salt stress [27]. Our earlier experiments *in vitro* have demonstrated that 1 M betaine stabilizes the oxygen-evolving complex of photosystem II by preventing the dissociation of extrinsic proteins and the disintegration of the manganese cluster [24]. It is likely that betaine not only acts as an osmoprotectant but also plays an essential role in the protection of the photosynthetic machinery against the effects of high salt.

The accumulation of betaine at about 80 mM in cells of *Synechococcus* sp. PCC 7942 that had been transformed with the *codA* gene significantly increased their tolerance to salt stress. This result is in agreement with recent results reported by Nomura *et al.* [21] who demonstrated that cells of *Synechococcus* sp. PCC 7942, after transformation with the *bet* genes of *E. coli* on plasmid vector pUC303, accumulated betaine at 45 mM and became salt-tolerant.

The photosynthetic activity of cells of strain PAMCOD declined for two days after the shift from NaCl-free medium to medium with 0.4 M NaCl (Fig. 7). However, the photosynthetic activity increased subsequently and then the cells started to proliferate again. These effects suggest that some lag time is necessary before the effectiveness of betaine in protecting the cells against the salt-induced inactivation of photosynthesis becomes apparent.

The present study demonstrates that the salt

tolerance of a cyanobacterium can be enhanced by genetic manipulation of the expression of choline oxidase. Because a similar system could operate in the salt tolerance of higher plants, it might be possible to improve the salt tolerance of such plants by similar manipulations of the expression of choline oxidase.

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## References

1. Arnon DI, McSwain BD, Tsujimoto HY, Wada K: Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. Biochim Biophys Acta 357: 231–245 (1974).
2. Boch J, Kempf B, Bremer E: Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. J Bact 176: 5364–5371 (1994).
3. Brouquisse R, Weigel P, Rhodes D, Yocum CF, Hanson AD: Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. Plant Physiol 90: 322–329 (1989).
4. Csonka LN: Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev 53: 121–147 (1989).
5. Elhai J, Thiel T, Pakrasi HB: DNA transfer into cyanobacteria. In: Gelvin SB, Schilperoort RA and Verma DPS (eds) Plant Molecular Biology Manual, pp. A12: 1–23 Kluwer Academic Publishers, Dordrecht, Netherlands (1991).
6. Falkenberg P, Str¿m AR: Purification and characterization of osmoregulatory betaine aldehyde dehydrogenase of *Escherichia coli*. Biochim Biophys Acta 1034: 253–259 (1990).
7. Gabbay-Azaria R, Tel-Or E, Schonfeld M: Glycinebetaine as an osmoregulant and compatible solute in the marine cyanobacterium *Spirulina subsulsa*. Arch Biochem Biophys 264: 333–339 (1988).
8. Incharoensakdi A, Takabe T, Akazawa T: Effect of betaine on enzyme activity and subunit interaction of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Aphanothece halophytica*. Plant Physiol 81: 1044–1049 (1986).
9. Ikuta S, Matuura K, Imamura S, Misaki H, Horiuti Y: Oxidative pathway of choline to betaine in the soluble fraction prepared from *Arthrobacter globiformis*. J Biochem 82: 157–163 (1977).
10. Ikuta S, Imamura S, Misaki H, Horiuti Y: Purification and characterization of choline oxidase from *Arthrobacter globiformis*. J Biochem 82: 1741–1749 (1977).
11. Imhoff JF: Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol Rev 39: 57–66 (1986).
12. Kaenjak A, Graham JE, Wilkinson BJ: Choline transport activity in *Staphylococcus aureus* induced by osmotic stress and low phosphate concentrations. J Bact 175: 2400–2406 (1993).
13. Lamark T, Kaasen I, Eshoo MW, Falkenberg P, McDougall J, Strøm AR: DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. Mol Microbiol 5: 1049–1064 (1991).
14. Landfald B, Strøm AR: Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J Bact 165: 849–855 (1986).
15. Ledeboer AM, Edens L, Maat J, Visser C, Bos JW, Verrips CT, Janowicz Z, Eckart M, Roggenkamp R, Hollenberg CP: Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. Nucl Acids Res 13: 3063–3082 (1985).
16. Mackay MA, Norton RS, Borowitzka LJ: Organic osmoregulatory solutes in cyanobacteria. J Gen Microbiol 130: 2177–2191 (1984).
17. Mamedov MD, Hayashi H, Wada H, Mohanty PS, Papageorgiou GC, Murata N: Glycinebetaine enhances and stabilizes the evolution of oxygen and the synthesis of ATP by cyanobacterial thylakoid membranes. FEBS Lett 294: 271–274 (1991).
18. Miyake C, Michihata F, Asada K: Scavenging of hydrogen peroxide in prokaryotic and eukaryotic algae: acquisition of ascorbate peroxidase during the evolution of cyanobacteria. Plant Cell Physiol 32: 33–43 (1991).
19. Mohanty P, Hayashi H, Papageorgiou GC, Murata N: Stabilization of the Mn-cluster of the oxygen-evolving complex by glycinebetaine. Biochim Biophys Acta 1144: 92–96 (1993).
20. Murata N, Mohanty PS, Hayashi H, Papageorgiou GC: Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen-evolving complex. FEBS Lett 296: 187–189 (1992).
21. Nomura M, Ishitani M, Takabe T, Rai AK, Takabe T: *Synechococcus* sp. PCC 7942 transformed with *Escherichia coli bet* genes produces glycine betaine from choline and acquires resistance to salt stress. Plant Physiol 107: 703–708 (1995).

22. Ohishi N, Yagi K: Covalently bound flavin as prosthetic group of choline oxidase. Biochem Biophys Res Commun 86: 1084–1088 (1979).
23. Papageorgiou GC, Fujimura Y, Murata N: Protection of the oxygen-evolving photosystem II complex by glycinebetaine. Biochim Biophys Acta 1057: 361–366 (1991).
24. Papageorgiou GC, Murata N: The unusually strong stabilizing effects of glycinebetaine on the structure and function in the oxygen-evolving photosystem II complex. Photosynth Res, in press.
25. Paul M: Limited N-terminal sequence analysis. Meth Enzymol 182: 602–613 (1990).
26. Rhodes D, Hanson AD: Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol 44: 357–384 (1993).
27. Robinson SP, Jones GP: Accumulation of glycinebetaine in chloroplasts provides osmotic adjustment during salt stress. Aust J Plant Physiol 13: 659–668 (1986).
28. Rozwadowski KL, Khachatourians GG, Selvaraj G: Choline oxidase, a catabolic enzyme in *Arthrobacter pascens*, facilitates adaptation to osmotic stress in *Escherichia coli*. J Bact 173: 472–478 (1991).
29. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
30. Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G: Purification and properties of unicellular blue-green algae (order Chroococcales). Bact Rev 35: 171–205 (1971).
31. Wall JS, Christianson DD, Dimler RJ, Senti FR: Spectrophotometric determination of betaines and ther quaternary nitrogen compounds as their periodides. Anal Chem 32: 870–874 (1960).
32. Wierenga RK, Terpstra P, Hol WGJ: Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$-fold in proteins, using an amino acid sequence fingerprint. J Mol Biol 187: 101–107 (1986).
33. Weretilnyk EA, Bednarek S, McCue KF, Rhodes D, Hanson AD: Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. Planta 178: 342–352 (1989).
34. Wood D, Darlison MG, Wilde RJ, Guest JR: Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*. Biochem J 222: 519–534 (1984).