Molecular Analysis of Genes involved in Carbohydrate Metabolism in the Desiccation-Tolerant Cyanobacterium Nostoc commune UTEX 584

by

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ABSTRACT

Synthesis of water stress proteins (Wsp) is induced upon repeated desiccation and rehydration of immobilized cells of the desiccation-tolerant cyanobacterium Nostoc commute UTEX 584 (Nostoc 584). The Wsp polypeptides synthesized and secreted by field material of N. commute have been extensively characterized and shown to exist as three isoforms with molecular masses of 33, 37 and 39 kDa. In order to understand the role of Wsp in the mechanism of tolerance to water stress an attempt was made to isolate the gene(s) that encodes Wsp in Nostoc 584. A polyclonal antibody raised against a mixture of the isoforms was used to screen expression libraries (phage and plasmid) of Nostoc 584 genomic DNA fragments. This work presents the analysis of clones, isolated from the expression libraries, which cross react with Wsp antiserum. Sequencing of the DNA from one of the cross reacting clones revealed an incomplete open reading frame (ORFA) that showed strong similarity to two fructose bisphosphate aldolases, CfxA and CfxB, from the photosynthetic purple non-sulfur bacterium, Rhodobacter sphaeroides. A promoter region present upstream of ORFA is recognized by RNA polymerase from E. coli. Further upstream of the promoter lies tmK encoding lysyl-tRNA, identified by evident similarity to
the corresponding gene from the chloroplast of the liverwort, *Marchantia polymorpha*. The remainder of the aldolase gene (*fba*) was isolated using the colony hybridization technique.

Sequence analysis of DNA from the second cross reactive clone revealed six ORFs (ORFs 1 through 6). The products of ORF1 and ORF2 are overproduced in this clone. The polypeptide encoded by ORF1 shows very strong cross-reactivity with the polyclonal Wsp antibody, whereas ORF2 does not. Database searches using the deduced amino acid sequences of the six ORFs have provided clues to the possible identities of these ORFs. ORF6 shows correspondence with a protein, in *Arabidopsis thaliana*, which is induced in response to cold, abscissic acid and water stress. The common feature shared by ORFs 1 to 5 is that the highest similarities are observed with enzymes involved in carbohydrate metabolism. ORFs 1 through 5 may possibly represent a novel cluster of genes that form all or part of an operon involved in the metabolism of carbohydrates in *Nostoc 584*.

Fructose bisphosphate aldolase (EC 4.1.2.13) is a key enzyme in carbohydrate metabolism, playing a role in glycolysis as well in the Calvin cycle of carbon dioxide fixation. The potential roles played by aldolase and the products of ORFs 1 through 5 in overall carbon metabolism of *Nostoc commune UTEX 584* are discussed.
FOR MY MOTHER AND FATHER,

SAVITRI AND RAMESH BABULKAR
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LIST OF ABBREVIATIONS

BSA: Bovine serum albumin
CAPS: 3-[cyclohexylamino]-1-propanesulfonic acid
CBB: Coomassie Brilliant blue
CTAB: Cetyl trimethylammonium bromide

DIG: Digoxigenin
dNTP: Deoxyribonucleoside triphosphate
EPS: Extracellular polysaccharides
F6P: Fructose-6-phosphate
FBP: Fructose-1,6-bisphosphate
IPTG: Isopropyl-β-D-thiogalactoside
LB: Luria Bertani
mA: Milliamperes
MDO: Membrane-derived oligosaccharides

Nostoc 584: Nostoc commune
UTEX 584

OD: Optical density (absorbance) at x nm

ONPG: ortho-nitrophenyl-β-D-galactoside
ORF: Open reading frame
PAGE: Polyacrylamide gel electrophoresis
PCR: Polymerase chain reaction
rpm: revolutions per minute
SDS: Sodium dodecyl sulfate
SSC: Standard saline citrate
TAE: Tris acetate EDTA
TBS: Tris buffered saline
TBST: Tris buffered saline with Tween-20
Tris: Tris(hydroxymethyl)aminoethane

Tween-20: Polyoxyethylene sorbitan monolaurate

v/v: volume to volume ratio
w/v: weight to volume ratio
Wsp: Water stress protein

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside
Introduction

Cyanobacteria comprise of a diverse group of prokaryotes capable of oxygenic photosynthesis. These organisms are evolutionarily ancient and are thought to be responsible for the progression of the earth’s atmosphere from the anaerobic to the aerobic state (Schopf and Walter, 1982). The phylogenetic relationship of cyanobacteria to chloroplasts has been well established (Bonen and Doolittle, 1976; Giovannoni et al, 1988). Cyanobacteria have a world-wide distribution. In their natural habitats, which span terrestrial to marine, their communities are often subject to extreme fluctuations in environmental conditions. Many cyanobacteria have adapted to, and thrive in ecological niches too harsh for most life forms. The filamentous, heterocystous cyanobacterium, *Nostoc commune* UTEX 584 (*Nostoc* 584) expresses a pronounced tolerance to water-stress (Potts and Bowman, 1985), and can withstand years of desiccation without loss of viability. Water is an indispensable component of all living cells. The ability to survive in the desiccated state confers ecological advantages to the limited number of organisms that are capable of it. However, the underlying mechanisms of the tolerance to water stress remain largely unknown. *Nostoc commune* has served as a useful model in the study of desiccation tolerance in prokaryotes, the subject of a recent review (Potts, 1994).

One of the responses of desiccation-tolerant cells to water deficit is the accumulation of large amounts of disaccharides such as trehalose or sucrose (Crowe et al, 1992). These disaccharides have been shown to stabilize membranes and proteins during desiccation (Crowe et al, 1987; Crowe and Crowe, 1986; Crowe and Crowe; 1992; Hoekstra et al, 1992; Hoekstra et al, 1991). The "water replacement hypothesis" provides an explanation for the protective effect of sugars such as trehalose (Clegg, 1986). According to the hypothesis polyhydroxyl compounds replace the water molecules around macromolecules,
thereby preventing damage during desiccation. Extracellular polysaccharides (EPS) in bacteria are widely believed to provide protection against drying (Ernst et al., 1987).

Periplasmic cyclic β-(1,2)-glucans, in members of the family *Rhizobiaceae*, are thought to be involved in adaptation to hypoosmotic conditions (Breedveld and Miller, 1994). The cyclic glucans are similar in structure to membrane-derived oligosaccharides (MDO) in *E. coli* which also accumulate in the periplasm and function in osmoregulation (Kennedy, 1982; Kennedy, 1987). The synthesis of polysaccharides in bacteria as a response to drying would necessitate regulation of the overall carbohydrate metabolism of the cell.

Survival of *N. commune* during multiple cycles of drying and rewetting may require coordinated interactions at the structural, physiological and molecular level (Potts, 1993). The glycan sheath, in which the filaments of *N. commune* are embedded, has been studied in detail (Hill et al., 1994b). The sheath is made up of complex polysaccharides and provides a physical barrier between the cells and their environment. It has been noted that during desiccation and rehydration the sheath undergoes various biochemical and physical changes and yet the cells that it surrounds appear to show no shrinkage or abnormalities at the ultrastructural level (Hill et al., 1994b). One of the first clues in deciphering the mechanism of desiccation tolerance in *N. commune* came from the discovery of copious amounts of water stress proteins (Wsp) in field material of *N. commune* (Scherer and Potts, 1989). These proteins are secreted into the extracellular glycan sheath together with UV-A/B-absorbing pigments (Hill et al., 1994a). A xylanase activity co-purifies with Wsp polypeptides, and the UV-A/B-absorbing pigments are found to associate with Wsp in the absence of salt (Hill et al., 1994a). Three Wsp polypeptides, with apparent molecular masses of 33, 37 and 39-kDa, have been purified from field material of *N. commune*. All three polypeptides cross react strongly with antiserum directed against the 39-kDa form of
Wsp purified by electroelution from SDS-PAGE gels (Scherer and Potts, 1989). The three forms of Wsp have identical amino-terminal sequences: Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln-X-Ile-Gln-Asn-Pro-Ser-Asn-Gly-Lys-Gln (X represents an amino acid residue that could not be identified in any of the forms of Wsp; Hill et al., 1994a; Scherer and Potts, 1989). Database searches using the consensus sequence showed similarities with enzymes involved in carbohydrate metabolism. The polypeptides showing the highest sequence similarity with the amino-terminal sequence of Wsp are N-acetyl-D-glucosamine binding lectin/chitinase and endo-1,4-β-D-glucosidase. The amino-terminal sequence determined for a 25-kDa proteolytic fragment of Wsp (a mixture of the three forms) was Glu-Ala-Arg-Val-Thr-Gly-Pro-Thr-Thr-Pro-Ile-Asp (Hill et al., 1994a). This internal sequence of Wsp showed 66% sequence identity with part of the sequence of a β-xylosidase, XynB, from the thermophilic anaerobe, *Caldocellum saccharolyticum* (Luthi et al., 1990). Rehydration fluids from field material of *N. commune* which are enriched in Wsp showed weak xylosidase activity but exhibited a strong xylanase activity associated with, but not directly attributable to, Wsp (Hill et al., 1994a). Both xylanases and xylosidases are involved in the hydrolysis of the backbone of β-1,4-xylans. Xylanases act upon internal xylosidic linkages whereas xylosidases attack the ends of xylooligosaccharides (Wong et al., 1988). Xylose-containing oligosaccharides are components of the polysaccharide core which is linked to one of the chromophores of the UV-A/B-absorbing pigment in *Nostoc commune* (Bohm, 1993). The relationship between the UV-A/B-absorbing pigments and Wsp and its associated xylanase activity in the extracellular glycan has yet to be resolved. Understanding the regulation of synthesis of the glycan sheath and its components, including Wsp and the UV-A/B-absorbing pigments, will contribute significantly to deciphering the mechanisms employed by *N. commune* in surviving desiccation. Attempts
to isolate the gene(s) encoding Wsp was undertaken as a first step towards this goal. The availability of Wsp antiserum and the information about the amino-terminal and internal amino acid sequence of Wsp suggested two approaches with which to achieve this objective:

1) Construction of an expression library of *Nostoc* 584 DNA for the purpose of screening with the Wsp antiserum and,

2) Synthesis of oligonucleotides based on the amino acid sequence and their use in the polymerase chain reaction (PCR) technique.

As part of the first approach, an expression library of *Nostoc* 584 in Lambda gt11 was constructed by Dr. S. Scherer and screened with the Wsp antiserum. A positive plaque was identified by its strong cross reaction against the polyclonal Wsp antibody (data not shown). Digestion of the DNA isolated from this recombinant with the restriction enzyme *Eco*RI yielded a 1124-bp fragment. Sequence analysis of this *Eco*RI-*Eco*RI fragment revealed the presence of an incomplete open reading frame (ORFA). A preliminary comparison of the sequence of ORFA with known sequences in the Genbank database suggested that *orfA* may encode the enzyme fructose bisphosphate aldolase. The major role played by fructose bisphosphate aldolase in carbohydrate metabolism, in general, and hexose metabolism in particular (see below), prompted further investigation of the putative aldolase from *Nostoc* 584. A more detailed discussion of ORFA can be found in subsequent sections.

Fructose-1,6-bisphosphate aldolase (Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase; EC 4.1.2.13) catalyzes the reversible aldol cleavage or condensation of fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. This enzyme catalyzes the fourth step in the Embden-Meyerhof pathway, following the key regulatory enzyme in the pathway, phosphofructokinase. It also
functions in the fixation of carbon dioxide, via the Calvin cycle, in plants and in photosynthetic bacteria. Thus, fructose bisphosphate aldolase plays a major role in the convergence of photosynthesis (anabolism) and glycolysis (catabolism).

Fructose-1,6-bisphosphate (FBP) is converted to fructose-6-phosphate (F6P) by the action of the enzyme fructose-1,6-bisphosphatase. F6P is a component of the hexose monophosphate pool which includes glucose-6-phosphate, glucose-1-phosphate, mannose-6-phosphate and mannose-1-phosphate. These five hexose phosphates are readily interconvertible (Zubay and Strominger, 1988). Mannose is one of the principal components of the glycan sheath of *N. commune* (Hill et al, 1994a). The disaccharides sucrose and trehalose function as compatible solutes in cyanobacteria and other organisms (Hershkovitz et al, 1991) during periods of water-stress. Fructose-1,6-bisphosphatase is a key enzyme in the biosynthesis of sucrose.

Two classes of fructose-bisphosphate aldolases, termed class I and class II, have been defined on the basis of structure and catalytic properties (Rutter, 1964; Morse and Horecker, 1968). The difference between the two classes lies in the mechanism employed to stabilize the carbanion intermediate. In class I enzymes a ketimine, formed between the carbonyl group of the substrate and a lysine residue in the active site of the enzyme, performs this function. Therefore, this class of aldolases is sensitive to inhibition by borohydride which can reduce the imine. A divalent transition-metal ion (usually Zn$^{2+}$ or Fe$^{2+}$) in the active site acts as an electron sink in class II enzymes. Metal chelators such as EDTA strongly inhibit the class II aldolases which are not affected by borohydride. The class I aldolases are usually tetramers consisting of identical polypeptide chains whereas the class II enzymes are generally dimeric. Within the same class, there is sequence similarity between the aldolases from diverse species. Sequence analysis at the amino acid level and the difference in properties of aldolases from the two classes prompted the suggestion that
each class of enzymes arose, independent of the other, from ancestral molecules that were unrelated (Rutter, 1964). This hypothesis was supported by the information available, at that time, about the phylogenetic distribution of the two classes of aldolases. Class I enzymes had been discovered only in eukaryotes (animals, plants, protozoans and algae) and class II enzymes had been reported only in prokaryotes (bacteria and cyanobacteria) and eukaryotic yeast and fungi. Rutter (1964) also proposed that class II enzymes were phlogenetically more ancient as compared to class I enzymes. Since then there have been reports of class I aldolases in a variety of prokaryotes (Lebherz and Rutter, 1973; Stribling and Perham, 1973; Fischer et al, 1982; Dhar and Altekar, 1986). Both classes of aldolases are found within each of the three primary kingdoms, Archaea, Eucarya and Bacteria; moreover, both classes of aldolases have been found to be expressed in the same organism (Marsh and Lebherz, 1992). Therefore, it can be postulated that both classes were present in the hypothetical progenote, making it difficult to predict which of the two classes is more primitive. Only a single type of aldolase activity is constitutively expressed by the majority of extant organisms. Marsh and Lebherz (1992) concluded that, as they evolved, most organisms have lost the capacity to express one or the other class of aldolases.

It has been reported that conditional lethal mutations in the gene for fructose bisphosphate aldolase, fba, in E. coli (ts8 and h8 mutations; Singer et al, 1991) and in orfY-ts (fba1) in Bacillus subtilis (Trach et al, 1988; Mitchell et al, 1992), inhibit the synthesis of stable RNA species. RNA synthesis is inhibited when the organisms are shifted to the nonpermissive temperature. Singer et al (1991) have demonstrated that the ts8 and h8 mutations inhibit initiation of transcription from stable RNA promoters and that this effect is seen in promoters subject to growth rate regulation. Synthesis of stable RNA is tightly linked to the growth rate of cells (Jinks-Robertson et al, 1983; Cole et al, 1987) and to the stringent response (Gallant, 1979; Cashel and Rudd, 1987) and in both cases is
regulated at the level of transcriptional initiation. Liebke and Speyer (1983) have reported that the mechanism of inhibition caused by the ts8 mutation is independent of the stringent response. The mechanism by which fba mutants inhibit synthesis of stable RNA is not fully understood. However, ts8 mutants can grow at the nonpermissive temperature on glycerol, pyruvate, a-ketoglutarate, succinate and 2-deoxyglucose, a non-metabolizable analog of glucose (Singer et al, 1991). Bock and Neidhardt (1966a; 1966b) have shown that the fructose-1,6-bisphosphate pool increases seven- to twenty-fold in the h8 mutant. Therefore it is likely that fructose-1,6-bisphosphate is directly or indirectly responsible for the inhibitory effect on stable RNA synthesis.

The prokaryotic cyanobacteria are known to express a class II aldolase. Cell-free extracts of the cyanobacteria Anabaena variabilis, Nostoc muscorum, Plectonema sp. and Anaebaenopsis sp. have been found to demonstrate class II aldolase activity (Willard and Gibbs, 1968). The presence of Fe^{2+} stimulates the activity of the aldolase of Anacystis nidulans. There are no reports in the literature of the primary sequence of a class II aldolase from cyanobacteria. In this study the gene for a class II fructose-1,6-bisphosphate aldolase has been isolated and sequenced. The information available from Nostoc 584 fba may provide another step in the understanding of the regulation of EPS synthesis and carbohydrate metabolism in this cyanobacterium.

The majority of the information available on Wsp, the glycan sheath and the UV-A/B-absorbing pigments has been elucidated from field material of Nostoc commune (Scherer and Potts, 1989; Hill et al, 1994a). Why then was the strain Nostoc 584 selected for isolation of wsp? The reason for this choice was three-fold. At the time this work was initiated the field material could not be grown in the laboratory as a pure culture. Nostoc 584, on the other hand, is a clonal axenic strain that can be maintained in liquid culture. The presence of a variety of contaminants in the field material precludes the use of a sensitive
technique such as PCR because the results obtained using this technique would be questionable. Another obstacle in working with the field material of *N. commune* is the difficulty in obtaining requisite amounts of purified high molecular weight DNA. All the problems associated with using the field material could be circumvented by using the strain *Nostoc 584* for the isolation of *wsp*. Extracts of *Nostoc 584* contain polypeptides in the range of 30- to 40-kDa that cross-react with the Wsp antiserum (Hill et al, 1994a). The results and analysis provided in this study are an attempt to understand the mechanisms involved in the desiccation-tolerance of the cyanobacterium, *Nostoc commune* UTEX 584.
Materials and Methods.

2.1 Bacterial strains and plasmids

The cyanobacterium Nostoc commune strain UTEX 584 (Nostoc 584) was obtained from the laboratory collection of Dr. M. Potts and was originally obtained from the University of Texas Culture Collection (Dr. M.P. Starr). The culture was rendered axenic by Ms. N.S. Morrison in Dr. M. Potts' laboratory.

The plasmids and E. coli strains that were used are listed in Table 1, and Table 2, respectively. The plasmid pCB267 and the E. coli strain TL73 were gifts from Dr. T.J. Larson. The E. coli strains PER1 and PER4 were gifts from Dr. P. Rouviere and Dr. C.A. Gross.

2.2 Media and Growth Conditions

Nostoc 584 was grown in airlift fermentors in the defined media BG11 or BG11o, in the presence or absence of combined nitrogen, respectively (Rippka et al, 1979). Sterile air, or a mixture of 5% CO₂/95% air (v/v) was sparged through the cultures during growth. Under the latter growth conditions 20-fold strength Na₂CO₃ (0.4 g l⁻¹) was added to the medium to buffer the pH.

E. coli strains were grown routinely in liquid culture, at 37°C, in Luria Bertani (LB) medium (Maniatis et al, 1989), supplemented with 100 µg ml⁻¹ ampicillin when required. LB agar plates, supplemented with antibiotic, X-gal and IPTG if appropriate, were used to maintain the strains on solid medium.
2.3 Isolation of genomic DNA

A modification of the procedure described by Murray and Thompson (1980) was used to isolate genomic DNA from Nostoc 584. Forty grams wet weight of Nostoc 584 was ground in the presence of liquid N₂ and glass beads using a mortar and pestle. One hundred milliliters extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1.25 % w/v hexadecyl trimethylammonium bromide (CTAB), 1% v/v β-mercaptoethanol) was added to the homogenized cells. The slurry was centrifuged at room temperature for 10 min at 2,600 x g, in a GSA rotor, to remove the debris. The supernatant fraction was incubated at 65 °C for 20 min and then frozen in liquid N₂ for 5 min. Additional CTAB was added to a final concentration of 2.25% w/v and the above step was repeated. After a further incubation at 65°C for 20 min, the supernatant fraction was extracted with an equal volume of chloroform/iso-amyl alcohol (24:1) and centrifuged for 5 min., at 2,600 x g, in a table-top centrifuge (IEC Centra-7R) at room temperature. The aqueous phase was extracted and centrifuged as above. An equal volume of ice-cold isopropanol was added to the aqueous phase which was then incubated at -20°C, overnight. The genomic DNA was spooled using a Pasteur pipette that had been drawn out in the shape of a hooked rod. The Pasteur pipette was then washed in 70% ethanol for 1 min. The excess 70% ethanol was removed and the spooled DNA was air dried. The DNA was allowed to dissolve in 20-ml TE buffer pH 7.5 (Maniatis et al, 1989), overnight, at 4°C. After the DNA had dissolved completely, 2-ml of ethidium bromide (10 mg ml⁻¹) and 70 gm of cesium chloride were added to the solution (ρ = 1.55) and the sample was centrifuged at 45,000 rpm for 42 hours, using a VTi 50 rotor in a Beckman ultracentrifuge. The DNA was recovered as described by Maniatis et al (1989).
2.4 Recombinant DNA Techniques

Standard protocols were followed for the construction of recombinant plasmids (Maniatis et al, 1989).

Isolation of Plasmid DNA: The Wizard Mini Prep columns (Promega Corporation) were used according to the manufacturer’s guidelines for isolation of plasmid DNA from 3 ml overnight cultures of the appropriate strain.

Digestion of DNA with Restriction Endonucleases: Genomic or plasmid DNA was digested with restriction endonucleases according to the specifications of the supplier (BRL).

Agarose Gel Electrophoresis and Purification of Restriction Fragments: The digested genomic or plasmid DNA was mixed with 0.1 volumes of 10-fold strength gel loading buffer II and was separated by electrophoresis on an agarose gel (0.7% - 2.0% w/v), at constant voltage, in TAE buffer (Maniatis et al, 1989). The linearized genomic (insert) DNA and plasmid (vector) DNA bands were excised from the gel using a sterile razor blade and the DNA was recovered by a freeze-thaw technique (Silhavy et al, 1984).

Ligation: If necessary, the digested plasmid was treated with calf intestinal alkaline phosphatase (Sigma), to dephosphorylate the vector (Maniatis et al, 1989). This procedure minimized self-recircularization of vector during ligation leading to decreased background during transformation. Vector and insert DNA were ligated using T4 DNA ligase, as recommended by the suppliers (BRL).

Transformation: E. coli strains were transformed by one of the two protocols described below.
Transformation of *E. coli* strains by the heat-shock method: Competent cells were either purchased (Maximum Efficiency DH10B competent cell, BRL) or were prepared in the laboratory, and they were transformed by an adaptation of Hanahan's procedure (Hanahan, 1985; Promega, 1991). The competent cells, at -70°C, were thawed on ice, gently mixed, and 100-μl aliquots were transferred into prechilled 1.2-ml cryovials (Nalgene). The ligation reaction was diluted 1:5 in TE buffer, pH 7.5 and 1 μl of the dilution was added to the competent cells. The tubes were mixed gently and incubated on ice for 30 min. The cells were heat-shocked for 45 sec in a 42°C water bath and then placed on ice for 2 min. SOC Medium (0.9-ml; Maniatis et al, 1989), was added to the cells and the mixture was shaken at 37°C, for 1 hr. The cells were transferred to 1.5-ml microcentrifuge tubes and centrifuged for 1 min in a microcentrifuge. Eight hundred microliters of the supernatant was discarded, the cells were resuspended in the remaining 200 μl and spread on an LB plate containing 100 μg ml⁻¹ ampicillin. The plates were incubated overnight at 37°C.

Transformation of *E. coli* strains by electroporation: *E. coli* cells were prepared for electroporation by the following procedure. Five milliliters SOB medium (Maniatis et al, 1989) was inoculated with a single colony of the appropriate *E. coli* strain and incubated overnight at 37°C, on a roller drum. A 1-l flask containing 250 ml SOB medium was inoculated with 2.5 ml of the overnight culture and this culture was incubated at 37°C, with vigorous aeration until an OD₅₅₀ value of 0.8 was reached. The cells were harvested by centrifugation at 2,600 x g in a GSA rotor, for 10 min, at 4°C. The cell pellet was washed in 250 ml of sterile, ice-cold washing buffer (10% glycerol: 90% distilled water, v/v). The cell suspension was centrifuged as above except that the time of centrifugation was 15 min. The supernatant fraction was discarded carefully and the
washing step was repeated. After discarding the supernatant the cells were resuspended in
the buffer that remained in the centrifuge bottle. Cells were either used immediately for
electroporation, or they were frozen in 200 µl aliquots in microcentrifuge tubes, in a dry
ice-ethanol bath. Frozen cells were stored at -70°C. Prior to electroporation the ligation
reactions were precipitated as described below. Two microliters of 3M NaOAc, pH 7.0
was added to 20 µl of ligation reaction in a microcentrifuge tube and mixed well. Fifty
microliters of 95% ethanol was added to precipitate the DNA and mixed. The tube was
spun in a microcentrifuge for 15 min at 4°C and the supernatant was discarded. The DNA
was washed with 60 µl of 70% ethanol and centrifuged as above. The supernatant was
discarded and the pellet was dried under a vacuum. The DNA pellet was resuspended in 20
µl half strength TE buffer pH 7.5.

The Cell-Porator Electroporation System and the Cell-Porator Voltage Booster (Life
Technologies Inc.) were used for electroporation according to the instructions of the
manufacturer. Twenty to Twenty five microliters of E. coli strain DH10B prepared
specifically for electroporation (thawed or freshly prepared) were transferred to a sterile,
prechilled microcentrifuge tube. One microliter of DNA (in half strength TE buffer) was
added to the tube and mixed. The cell-DNA mixture was suspended between the electrode
bosses of a prechilled Disposable Micro-Electroporation Chamber (a 0.15-cm electrode gap
is generated when the cap is sealed) making sure to avoid air bubbles. The chamber rack
was placed in the chamber safe that had been filled with a slurry of ice and water. The
loaded chamber was placed in one of the four slots in the chamber rack. Cells were
electroporated at pulse settings of 2.4 kV, 4kΩ (field strength of 16 kV cm⁻¹ and a time
constant of 6 milliseconds). After electroporation the cells were allowed to remain in the

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chamber for 5 min. To permit uptake of DNA the cells were then diluted with 1.5 ml of SOC medium and transferred to sterile tubes (13 x 100 mm) fitted with metal caps and shaken for 1 h, at 37°C. At the end of the incubation the cells were plated on LB agar containing 100 µg ml\(^{-1}\) ampicillin and the plates were incubated at 37°C overnight.

2.5 Construction of Genomic Libraries

Plasmid libraries: Two vectors were used in the construction of genomic libraries. The expression vector pTre99A was employed to create an expression library that could be screened immunologically, whereas the plasmid vector pUC18 was used to generate a library to be screened by hybridization techniques. The plasmid vector pTre99A was selected for the strong promoter (trc) which directs transcription of inserts cloned in the multiple cloning site. The trc promoter is so called because it contains the -35 region of the trp promoter and the -10 region and operator of the lacUV5 promoter. The hybrid is much more efficient than the individual promoters (three times as compared to trp and eleven times as compared to the lacUV5 promoter). The lacI\(^{a}\) gene in the vector facilitates induction in any E. coli strain. IPTG (1-5 mM) was used to induce transcription from the trc promoter. A basal level of transcription is possible, in the absence of inducer, because of the strength of the promoter. The E. coli strain DH10B was chosen to enable cloning of Nostoc 584 genomic DNA which exhibits a high degree of methylation (Jager and Potts, 1988).

Genomic DNA from Nostoc 584 (50 µg) was digested with the restriction endonucleases EcoRI or HindIII according to the specifications of the supplier (BRL). The digested DNA was purified by successive extractions with 1) an equal volume of equilibrated phenol, 2) an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1)
and 3) an equal volume of chloroform/iso-amyl alcohol (24:1). The DNA was subsequently precipitated with 2.5 volumes of ice-cold 95% ethanol after the addition of 0.1 volumes of 3 M sodium acetate, pH 5.2. Digestion and dephosphorylation of vector DNA, ligation of vector and insert DNA, and transformation of appropriate *E. coli* cells were performed as described (Section 2.4). Following ligation and transformation the transformants were plated out on LB agar plates containing 100 μg ml⁻¹ ampicillin.

**Phage library** : The replacement vector, Lambda FIX®II (Stratagene), was used to create a library of genomic DNA from *Nostoc 584*. The strains chosen to serve as hosts were XL1-Blue MRA and XL1-Blue MRA (P2) (see Table 1) because of their ability to stabilize clones containing methylated DNA.

**Preparation of host bacteria** : A single colony of the strain XL1-Blue MRA (P2) was inoculated into 25 ml of LB medium (supplemented with 0.2% w/v maltose and 10 mM MgSO₄) and the culture was grown at 37°C, on a shaker, until the OD₆₀₀ reached a value of 0.8-0.9. The cells were harvested by centrifugation at 2,000 rpm (GSA rotor), for 10 min and resuspended in 12.5 ml of sterile 10 mM MgSO₄. The OD₆₀₀ of the cell suspension was adjusted to a value of 0.5 with sterile 10 mM MgSO₄. This preparation of plating bacteria was stored at 4°C for a maximum of 48 hours before use.

**Preparation of the insert** : Genomic DNA from *Nostoc commune* UTEX 584 (50 μg) was partially digested with the restriction endonuclease *BamHI* and the cohesive ends were partially filled in with dATP and dGTP as recommended by the supplier (Lambda FIX®II/XhoI Partial Fill-In Vector Kit; Stratagene). The inserts were purified by organic extraction and precipitated as described above. Equimolar amounts of insert and Lambda
FixII vector arms were ligated using T4 DNA ligase according to the suggestions of the manufacturer (BRL).

Packaging of recombinant phages, titering and amplification the phage library: The Gigapack® II Gold packaging extracts (Stratagene) were used for packaging the recombinant phages. The titering and amplification of the phage library was performed as described by the manufacturer of the Lambda FIX®II/Xho I Partial Fill-In Vector Kit (Stratagene).

2.6 Colony Lifts and Screening of Expression Library

A sterile nitrocellulose filter (filter A; 0.45-μm pore size, Schleicher and Schuell) was labeled and marked and laid with labeled side down on a LB (containing 100 μg ml⁻¹ ampicillin) plate supporting transformants (original plate). The lid was replaced and the plate was inverted. Marks were made on the plate to match those on filter A. Next, filter A was carefully removed and placed, colony-side-up, on a fresh LB (containing 100 μg ml⁻¹ ampicillin) agar plate which was incubated in an inverted fashion at 37°C, for 4-6 h. The original plate was wrapped with Parafilm and stored at 4°C. At the end of the incubation at 37°C filter A was used for making replicas or stored, at 4°C, along with the plate (if there was a delay in making replicas).

One ml 5 mM IPTG was pipetted into a sterile Petri dish. A sterile filter (filter B) was placed in the Petri dish and allowed to soak in the IPTG solution. Filter B was allowed to dry by placing it on a sterile piece of filter paper (Whatman No. 3). Filter A was placed colony side up on sterile filter paper. Filter B was labeled and placed labeled side down on
Filter A. A piece of filter paper was placed on top of the filter sandwich and uniform pressure was applied to ensure efficient replication. The filter sandwich was turned upside down and a razor blade was used to mark filter B to match filter A. The filters were carefully separated and filter B was placed colony-side-up on a fresh LB agar plate (containing 100 μg ml\(^{-1}\) ampicillin) which was incubated inverted, at 37°C, for 4-6 h. Filter A was either used to make more replicas, or transferred colony-side-up to a LB agar plate (containing 100 μg ml\(^{-1}\) ampicillin and 25% glycerol) and incubated, at 37°C, for 1 h. After incubation the plate containing filter A was wrapped in Parafilm and stored inverted, at -20°C, in a plastic bag (glycerol stock). If more than two replicas were made, a longer incubation at 37°C was necessary to regenerate colonies.

Filter B was placed colony side up for 10 min on gel blot paper saturated with binding buffer (25 mM NaCl, 5 mM MgCl\(_2\), 0.5 mM DTT, 25 mM HEPES pH 7.9, 6 M guanidine HCl, 10 mM EDTA; Vinson et al, 1988). The filter was then placed colony side up on gel blot paper saturated with CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer. After 2 min the filter was transferred to a Petri dish and washed 3 times with CAPS buffer, 2 min for each wash. Following the third wash the filter was blocked with 1% BSA in TBST (w/v) for 20 min on the shaker. The filter was rinsed 3 times with TBST and subsequently incubated in primary antibody overnight (Wsp rabbit antiserum, 1:2,000 in TBST; Scherer and Potts, 1989). Before incubation in secondary antibody (Goat Anti-Rabbit IgG-Horseradish Peroxidase conjugate, Bio-Rad; 1:1,000 in TBST) for 2 h the filter was washed twice in TBST for 10 min each. This was followed by thorough rinsing in TBST and 2 washes, 5 min each, with TBS. The solutions for color development were freshly prepared. Chloronaphthol (30 mg) was dissolved in 10 ml of ice cold methanol and mixed with 50 ml TBS containing 30 μl of 30% H\(_2\)O\(_2\) immediately prior to use. Filter B
was immersed in this mixture and shaken till required intensity of color was achieved (1 - 10 min). Color development was stopped at the appropriate time by washing with distilled water. Positive colonies are purplish blue in color.

2.7 DNA Labeling and Colony Hybridization

The steroid hapten, digoxigenin, was used to generate non-radioactively labeled DNA probes (Martin et al., 1990). Double stranded DNA probes were labeled with digoxigenin-11-dUTP by the random primed method as described by the supplier (Genius 1 DNA Labeling and Detection Kit, Boehringer Mannheim Biochemicals).

Colony lifts and replicas were made as described in Section 2.6. The filter was placed colony side up on gel blot paper saturated with denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 15 min, at room temperature. The filters were then incubated on gel blot paper saturated with neutralization solution (1 M Tris-HCl, pH 8.0, 1.5 M NaCl) for 15 min. The DNA was fixed by baking the filters at 80°C, for 2 hours, in a vacuum oven. To avoid a high background the cell debris was removed by incubating in standard prehybridization solution (5-fold strength SSC, 1% w/v Blocking reagent from Genius 1 Kit, 0.1% N-lauroylsarcosine, 0.2% SDS) with shaking for 1 hour at 65°C before wiping the filters gently with a wet paper towel. The filters were prehybridized with gentle shaking for 2 hours at 65°C in a sealed bag containing enough standard prehybridization solution to cover all the filters. After the prehybridization solution was discarded it was replaced with hybridization solution (heat denatured, DIG-labeled double stranded probe added to standard prehybridization solution at a concentration of 20 ng ml⁻¹). The bag was resealed and incubated, at 65°C, overnight. The hybridization solution can be reused and was stored at -20°C, in a disposable 50-ml centrifuge tube. The filters were washed twice in double strength wash solution (double strength SSC containing 0.1% SDS) at 65°C for 5
min each, to remove unbound probe. This was followed by two washes of 15 min each in half strength wash solution (half strength SSC containing 0.1% SDS) at the same temperature. The filters were then ready for colorimetric detection which was performed at room temperature. The filter was equilibrated in filtered Genius buffer 1 (150 mM NaCl, 100 mM Tris-HCl pH 7.5) for 1 min prior to the blocking step. After transfer to a bag, the filter was blocked by gentle shaking in Genius buffer 2 (150 mM NaCl, 100 mM Tris-HCl pH 7.5, 2% w/v blocking reagent [Genius 1 Kit]) for 30 min. The blocking solution was then poured out and replaced with the anti-digoxigenin (alkaline phosphatase conjugate) antibody solution, which was diluted 1:5,000 in Genius buffer 2, for 30 min. The filters were then washed twice, 15 min per wash, in 100 ml Genius buffer 1, to remove unbound antibody. Before color development Genius buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂) was used to equilibrate the filters for 2 min. The color substrate solution was prepared fresh by mixing 45 µl nitroblue tetrazolium salt (75 mg ml⁻¹ in 70% v/v dimethylformamide) and 35 µl 5-bromo-4-chloro-3-indolyl phosphate (toluidinium salt; 50 mg ml⁻¹ in 100% dimethylformamide) in 10 ml of Genius buffer 3 and protected from direct light before use. Genius buffer 3 was poured off and the color substrate solution was added to the filters. The filters were incubated in the dark, in a sealed plastic bag, without shaking. The color reaction was stopped by rinsing the filters with sterile distilled water.

2.8 DNA Sequencing and Searches of Databases

The dideoxy chain termination method (Sanger et al., 1977) was used for determining the sequence of DNA from the recombinant clones of interest. DNA sequencing was done by Monika Ehling-Schultz and Elke Weiβner-Gunkel in the
laboratory of Dr. S. Scherer, at Technische Universität München, Freising, Germany. The nucleotide sequence obtained was compared to sequences in the Genbank and Swiss-Prot databases in an attempt to search for similarity to known sequences. The program PCGENE was used for manipulation of the nucleotide sequence and the program I FIND was used to search for similarity to the sequences in the databases.

2.9 Polymerase Chain Reaction

The oligonucleotide primers used in PCR are shown in Fig 9 and were synthesized in the laboratory of Dr. T.J. Larson. The primers 97015 and 97044 were designed on the basis of the partial sequence of amino acids at the amino-terminus of Wsp. The amino-terminal sequence of a 25-kDa fragment of Wsp generated by proteolytic digestion with endo Glu-C was used to design the primers 39224 and 39235. To account for degeneracy either a mixture of the relevant bases was used (97015 and 39224) or the base inosine was used in the third position (97044 and 39235). The standard PCR buffer contained 20 mM Tris-HCl, pH 8.3, 25 mM KCl and 1.5 mM MgCl₂ and 50 μM of each dNTP (pH 7.0).

The total reaction volume of 100 μl included 20 pmol of each primer and 2 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus). Genomic DNA from Nostoc 584 was used as the template. The standard temperature profile was 94°C (denaturing), 65°C (annealing) and 72°C (extension) for 35 cycles. In an effort to optimize the conditions for amplification some of the parameters were varied; annealing temperature (55 - 72°C), MgCl₂ (1 - 3 mM), dNTP (50 - 250 μM), primer concentration (20 pmol - 1,000 pmol) and template DNA (2 ng- 750 ng).
2.10 SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Separation of proteins was achieved by analytical SDS-PAGE (Laemmli, 1970) on 1.5-mm minigels in a Mighty Small II SE 250 chamber (Hoefer Instruments, San Francisco). The discontinuous gel consisted of a 5% w/v stacking gel and a 12-15% w/v running gel and was electrophoresed using a Tris-Glycine running buffer (25 mM Tris-HCl, 200 mM glycine, and 3.5 mM Sodium dodecyl sulfate [SDS]). A sample loaded on the SDS-gel was prepared as follows: 1.5-ml culture volume of cells was centrifuged in a microcentrifuge for 1 minute. The supernatant was discarded and the cell pellet was resuspended in 150-μl sterile distilled water. An equal volume of double strength denaturing sample buffer was added to the cells and mixed by vortexing the sample. The samples were then incubated in a boiling water bath for 10 minutes before loading on to the SDS-gel. Molecular weights of sample proteins were estimated by concurrent electrophoresis of prestained low-molecular weight markers (Bio-Rad Laboratories). Following separation, the proteins were transferred to a polyvinylidene difluoride membrane using CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer at pH 10.3, at 450 mA for 20-25 min. The Wsp (rabbit) antiserum was utilized for immunodetection using standard techniques. Membranes with transferred proteins were blocked for 20 min in 1% w/v BSA in TBST, rinsed for 1 min in TBST and then incubated in Wsp antibody (primary antibody; diluted 1:2,000 in TBST) overnight. The membranes were washed twice with TBST for 10 min each before being incubated with the secondary antibody (Goat-Anti-Rabbit IgG Horseradish peroxidase conjugate [Bio-Rad Laboratories]; diluted 1:1,000 in TBST) for 2 hours. This was followed by 3 washes with TBST (5 min each)
and 3 washes with TBS (5 min each) prior to color development. The solutions for color development, solution A (30 μl of 30% H₂O₂ in 50 ml TBS) and solution B (30 mg of 4-chloro-1-naphthol in 19 ml of ice-cold methanol), were mixed immediately prior to use. Membranes were immersed in the above solution until the required intensity of color was reached (5-10 min) and then they were rinsed with distilled water to stop the color reaction.

2.11 Promoter Activity Assay

The promoter probe vector, pCB267 contains two divergently arranged promoterless reporter genes, lacZ and phoA, separated by a multiple cloning site (Schneider and Beck, 1987). Promoter activity is demonstrated by the construction of transcriptional fusions that direct transcription of lacZ or phoA. The region upstream of the translation start codon of fba was assayed for promoter activity (see Fig. 5). The vector pCB267 was prepared by digesting with the enzyme BamHI followed by dephosphorylation with the enzyme calf intestinal alkaline phosphatase. The 1.1-kb EcoRI-EcoRI insert in pVJ004 (see Figs. 1 and 5) was partially digested with the restriction endonuclease Sau3AⅠ. The DNA fragments generated by the partial digestion were resolved by agarose gel electrophoresis using a 1.0% (w/v) agarose gel. The DNA band corresponding to the 537-bp fragment (see Fig. 5) was excised from the gel and purified as described in Section 2.4. The purified 537-bp fragment was cloned into the BamHI site of pCB267. The recombinants were transformed into E. coli strain DH5αF" and plated on LB agar containing X-gal. The appearance of blue colonies indicates that the transcriptional fusion was successful. A recombinant plasmid isolated from a blue colony was analyzed.
for promoter activity. The *E. coli* strain TL73 (ΔlacZ) was transformed with this recombinant plasmid and assayed for β-galactosidase activity as described below.

The formation of the product *o*-nitrophenol (yellow) from the substrate ONPG (colorless) was measured spectrophotometrically at a wavelength of 420 nm. A single colony from a fresh plate was inoculated into YT medium containing 100 mg ml\(^{-1}\) ampicillin and grown overnight at 37°C on a roller drum. The overnight cultures were inoculated into fresh YT medium containing 100 μg ml\(^{-1}\) ampicillin and grown to OD\(_{578}\) of 0.4 - 0.5. The cells were harvested and resuspended in Z buffer (60 mM Na\(_2\)HPO\(_4\), 40 mM NaH\(_2\)PO\(_4\), 10 mM KCl, 1 mM MgSO\(_4\), 50 mM β-mercaptoethanol) in a final volume of 0.8 ml. The cells were permeabilised by the addition of 15 μl of 0.1% SDS and 30 μl of chloroform and incubated at 30°C for 5 minutes. To start the reaction, 0.16 ml ONPG (4 mg ml\(^{-1}\) in 0.1 M phosphate buffer) was added and the cells were incubated at 30°C. After a yellow color developed the reaction was stopped by adding 0.5 ml of 1 M Na\(_2\)CO\(_3\). After centrifuging the reactions to remove the cell debris the absorbance was measured at a wavelength of 420 nm against a blank containing Z buffer and ONPG only. The following formula was used to calculate β-galactosidase activity in Miller units (Miller, 1972):

\[
\text{Miller units} = \frac{(\text{OD}_{420} \times 800)}{(\text{OD}_{578} \times \text{v} \times \text{t})}
\]

\( \text{v} = \text{volume of culture in ml} \)

\( \text{t} = \text{time of reaction in minutes} \)

2.12 Aldolase Assay
The enzymatic activity of aldolase was measured using an aldolase kit (Sigma Diagnostics®, Procedure No. 752). The assay is a modification of the techniques described by Sibley and Lehninger (1949) and by Wolf (1972). Fructose-1,6-bisphosphate aldolase catalyzes the following reaction:

Fructose-1,6-bisphosphate → Glyceraldehyde-3-phosphate (GAP) + Dihydroxyacetone phosphate (DHAP)

GAP is converted to DHAP by the enzyme triose phosphate isomerase. The free triose which results from the hydrolysis of DHAP, is converted partially to hydroxypyruvic aldehyde in the presence of Tris and alkaline conditions. The aldehyde reacts with 2,4-dinitrophenylhydrazine to form the hydrazone. The purple color formed by this product, upon addition of alkali, is proportional to the activity of the aldolase.

The strains to be assayed were grown by inoculating a single colony of the appropriate strain in 5 ml LB medium (containing 100 μg ml⁻¹ ampicillin) and incubating overnight, at 37°C, on a roller drum. The overnight cultures were diluted (1:100) into 25 ml fresh LB medium (containing 100 μg ml⁻¹ ampicillin) and grown as before, for 3 hours. An aliquot of cells (10 ml) was harvested and resuspended in 10 ml M9 medium (uninduced sample). IPTG (5 mM final concentration) was added to the remainder of the cells which were grown further for 3 hours. A 10-ml aliquot of these cells was harvested and resuspended in 10 ml M9 medium (induced sample). The OD₆₀₀ value of these cell suspensions was recorded. The cells were permeabilised by the addition of 15 μl of 0.1% SDS and 30 μl of chloroform to 1.0-ml of the cell suspensions. The permeabilised cells (0.1 ml) were used for assaying aldolase activity. The assay was performed in triplicate according to the specifications of the supplier except that the volumes of the reagents used
were scaled down to 50% of the recommended volumes. The purple color was measured spectrophotometrically at 560 nm. Aldolase activity was calculated as described by Singer et al (1991).

\[ \text{Arbitrary units} = \frac{[1000 \times \text{OD}_{560} \times \text{dilution factor}]}{[\text{OD}_{600} \times 0.1 \text{ ml}]} \]

The *E. coli* strain PER1 is a temperature-sensitive mutant which synthesizes a heat labile form of fructose-1,6-bisphosphate aldolase. PER1 grows well at 37°C, growth is poor at 42°C and this strain does not show any growth at 44°C. The mutation in PER1 has been shown to be dominant to wild-type (Singer et al, 1991). The wild-type strain, PER4, grows well at all three temperatures. Each of the two strains was transformed with the plasmids pVJ004 and pVJA52. The strains were also transformed with vector (alone) for use as a negative control.
Results

3.1 Isolation and sequencing of fba.

A portion of the gene for fructose bisphosphate aldolase in Nostoc 584, fba, was isolated as a result of screening an expression library of Nostoc 584 genomic DNA in the vector λgt11. The remainder of fba was isolated in the form of two overlapping fragments, by the technique of “chromosome walking” (see Fig. 1). Analysis of the nucleotide sequence of the 1.1-kb insert in pVJ004 (see Fig. 1) revealed the presence of one incomplete ORF, consisting of 181 amino acids, starting at position 582 in the DNA sequence (see Fig. 2). A potential ribosome binding site (AGGAGG) was present at position 569-574, 7 nucleotides upstream of the putative translation start codon (ATG). A preliminary comparison of the deduced amino acid sequence of the ORF with the sequences in the Genbank database showed a striking similarity with two fructose-1,6-bisphosphate aldolases from the photosynthetic, purple non-sulfur bacterium, Rhodobacter sphaeroides. (see Fig 3; Chen et al., 1991; Gibson et al., 1991). On the basis of this strong similarity the gene was identified as coding for the enzyme fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase (EC 4.1.2.13) and was termed fba. The fructose bisphosphate aldolase polypeptides in R. sphaeroides, CfxA and CfxB, consist of 359 and 354 amino acids respectively. The technique of DNA hybridization was employed to isolate the remainder of fba from Nostoc 584.

A 400-bp HindIII-EcoRI fragment (see Fig 1) was used as a probe to isolate the rest of the fba gene of Nostoc 584. The probe was labeled non-radioactively with digoxigenin-11-dUTP by the random primed method. A library of HindIII fragments of Nostoc 584 genomic DNA was screened with this probe using the colony hybridization
technique. A positive clone, VJA5, was identified and analyzed further. Digestion of the recombinant pVJA5 yielded multiple HindIII-HindIII fragments ranging in size from 726 bp to 2.2 kb. Restriction analysis and secondary screening identified the 726-bp HindIII-HindIII fragment to be the fragment that overlaps the 1.1-kb EcoRI-EcoRI fragment that contains fba (see Fig. 1). Since fba was still incomplete, another probe, a 326-bp EcoRI-HindIII fragment generated from pVJA5 (see Fig 1), was used to isolate the remainder of fba. Screening of the library of EcoRI fragments of Nostoc 584 DNA by colony hybridization yielded a single positive clone VJA53 (see Fig. 1). Restriction analysis of plasmid DNA from VJA53 revealed the presence of a single 2.0 kb EcoRI-EcoRI fragment. The sequence of this 2.0 kb insert was partially determined and the translation stop codon for Fba identified. The entire nucleotide sequence of fba and the deduced amino acid sequence of Fba are shown in Fig. 2.

3.2 Sequence analysis of fba and flanking region.

The preliminary identification of fba was confirmed by the isolation and sequencing of the complete gene from Nostoc 584. The strong similarity of Fba to the two fructose bisphosphate aldolases, CfxA and CfxB, is evident over its entire length. Comparison of the deduced amino acid sequences of fba, cfxA and cfxB are shown in Fig. 3. The Fba and CfxA polypeptides consist of 359 amino acid residues each whereas the CfxB polypeptide is slightly smaller at 354 residues. Alignment of the amino acid sequence of Fba with those of CfxA and CfxB shows 63% identity in both cases. If conservative amino acid substitutions are considered the similarity is extended further to 78% (with CfxA) and 79% (with CfxB). Amino acids were grouped together based on similarity of structure and the codons that represent them. For example, in the group "PAGST", alanine and glycine are
similar in structure; all five amino acids have a four-fold redundancy such that a change in the nucleotide in the “wobble” position does not change the amino acid coded. The amino acids proline, alanine, serine and threonine all have cytosine as the base in the middle position of the codon. Therefore, a change in the 5' nucleotide would result in interconversion within the group. There are no gaps required in the alignment of the three polypeptides. Comparison of CfxA and CfxB at the amino acid level shows 81% identity (Chen et al., 1991). The identification of cfxA as the gene encoding aldolase was deduced from sequence similarity with fba in E. coli - a gene which codes for a class II fructose-1,6-bisphosphate aldolase (Gibson et al., 1991). The Nostoc 584 fba also shows similarity with the corresponding gene in E. coli but to a lesser extent than with the genes in R. sphaeroides. There is considerable similarity between Nostoc 584 Fba and the product of orfY-tsr (also referred to as fba1) from Bacillus subtilis (Mitchell et al., 1992a; Mitchell et al., 1992b). Amino-terminal sequences of class II aldolases from Saccharomyces cerevisiae and Bacillus stearothermophilus (Alefounder et al., 1989) are also similar to the corresponding sequences from Fba of Nostoc 584.

Two signature patterns have been described for class II fructose bisphosphate aldolases (Bairoch and Bucher, 1994; Fig. 4). One of the conserved regions in these enzymes, which is located in the amino-terminal half, includes two histidine residues that are involved in binding zinc (Berry and Marshall, 1993) which is required for aldolase activity. The second motif includes a pair of acidic amino acid residues and is found in the carboxy-terminal half of the polypeptides. Both the aldolase consensus patterns are present in Nostoc 584 Fba.

Analysis of the nucleotide sequence immediately upstream of the translation start codon of Fba suggested the presence of a promoter for fba. The putative promoter region of fba contains the hexamers, TATTTA at the -10 region (position 431 to 436; Figure 2)
and TAGAGA at the -35 region (position 411 to 416) which are placed 14 nucleotides apart. The presence of promoter activity was demonstrated as described in section 3.3 below.

Further upstream of the putative promoter is a stretch of 72 nucleotides that has been termed trnK, the gene encoding lysyl-tRNA. The basis for this identification is the similarity that is exhibited by trnK with the corresponding gene in chloroplasts of the liverwort Marchantia polymorpha (Ozeki et al., 1987; Accession # M20959). The two genes share 89% identity (see Fig. 8). The codon recognized by lysyl-tRNA is AAA. Therefore the sequence of the anticodon is TTT (or UUU in the RNA product).

Comparison of the sequence of trnK from Nostoc 584 with corresponding sequences from E. coli (Accession # X52796) and the alga Codium fragile (Accession # M26735) indicated that the similarity is not as strong as it is with trnK from liverwort (results not shown).

An observation was made regarding the stability of the plasmid construct pVJ004. This plasmid was found to be unstable in some strains of E. coli (LE392, JM109, DH5αF'). However, it was observed that pVJ004 was stable in BL21(DE3), strain VJ004B (see Tables 1 and 2). The reason for the instability of pVJ004 is unclear but it may be due to certain sequence patterns present in the 1.1-kb insert. This is discussed in further detail in Section 4.1.

### 3.3 Promoter Activity Assay

The region upstream of the ATG start codon of fba was tested for promoter activity. The 537-bp Sau3aI- Sau3aI fragment, containing the putative promoter region, was cloned into the BamHI site of the promoter probe vector pCB267 as described in section 2.11. This fragment contains the putative promoter region of fba as well as a portion of the
coding region of \textit{fba} (see Fig. 5) The recombinant, pVJ008, was transformed into strain TL73 (\textit{lac'}) generating strain VJ008T to facilitate the assay of \(\beta\)-galactosidase activity. The strain used as a negative control for the assay was CB267T which was constructed by transforming pCB267 into the strain TL73. The results of the assay are shown in Fig. 6 and clearly demonstrate promoter activity in strain VJ008T.

\textbf{3.4 Expression of \textit{Nostoc} 584 \textit{fba} in \textit{E. coli}}

The entire \textit{Nostoc} 584 \textit{fba} was isolated in the form of three overlapping fragments by the technique of 'chromosome walking' (see Section 3.1). A number of subclones contain fragments of \textit{fba} (see Fig. 1; Table 1). The clone VJ004 contains the 1.1-kb \textit{EcoRI-}
\textit{EcoRI} fragment that carries \textit{trnK} and approximately half of the \textit{Fba ORF}. The parent plasmid of pVJ004, pGEM-4, contains a T7 promoter at one end of the MCS. It was determined, by restriction analysis, that \textit{fba} was in the correct orientation with respect to the T7 promoter in the strain VJ004B. An attempt was made to overexpress \textit{fba} by induction of VJ004B with IPTG. SDS-PAGE of total cellular proteins of uninduced and induced cultures of VJ004B showed that the truncated Fba was present in the induced, cultures (data not shown) at the expected molecular mass of 20 kDa. However, \textit{fba} was not overexpressed in VJ004B and Fba was observed as a barely discernible band after staining with Coomassie Brilliant Blue (CBB). No corresponding band was noticeable in the uninduced cultures. Since the lysyl-tRNA gene may have been a potential obstacle to overexpression of the truncated Fba, a shorter fragment was cloned into pGEM to yield the recombinant plasmid pVJ007 (see Fig 1). Induction of the strain VJ007 with IPTG followed by SDS-PAGE and CBB staining yielded results comparable to those obtained with VJ004B (data not shown).
Similar experiments with the strain VJA52 (see Fig. 1) showed massive overexpression of the truncated \textit{fba} in the induced cultures (data not shown). The parent plasmid in VJA52 is \textit{pTrc99A}. The molecular mass of the shorter Fba was approximately 32 kDa which agrees well with the expected value. The truncated polypeptide has 68 amino acids missing at the carboxy-terminus. However it does contain both the signature sequences associated with class II aldolases.

Attempts to construct a recombinant containing the entire Fba have been unsuccessful so far, although it appears that the ligations were successful. It is possible that overexpression of the full-length Fba is toxic in some way to the host cell.

\subsection*{3.5 Assessment of complementation of an \textit{E. coli} fba mutant}

The \textit{E. coli} strain PER1 contains a temperature-sensitive mutation in \textit{fba}. This mutant was used to assess the ability of truncated forms of \textit{Nostoc 584} to complement the mutation. Aldolase activity of uninduced and induced samples was assayed as described in Section 2.12. Transformation with the plasmid pVJ004 had no effect on the aldolase activity of either the mutant, PER1 or wild-type strain, PER4 (data not shown). This is not surprising since the Fba of pVJ004 is only half the normal length and is not present in large enough quantities to overcome the dominant mutation. In similar experiments with the plasmid pVJA52 it was observed that induction of strains with IPTG resulted in lower aldolase activity as compared to uninduced strains (see Fig. 7). In the wild-type strain, PER4-A52, the aldolase activity was down to approximately 68\% of normal value and in the mutant PER1-A52, it was approximately 58\% of normal value. PER4 and PER1 strains not containing the plasmid pVJA52 showed aldolase activities similar to those of the uninduced cultures of PER4-A52 and PER1-A52 (data not shown).
The temperature-sensitive phenotype of PER1 was not relieved by either pVJ004 or pVJA52.

3.6 Use of PCR to isolate wsp

Oligonucleotide mixtures were synthesized based on portions of the internal and N-terminal amino acid sequences that were determined for Wsp. These degenerate oligonucleotides were used as primers in PCR experiments, along with *Nostoc* 584 genomic DNA, in an effort to isolate wsp. Numerous attempts to amplify wsp by the technique of PCR have been unproductive. Varied reaction conditions have been utilized in an effort to optimize the procedure for isolation of wsp from *Nostoc* 584 (data not shown). Genomic DNA from *Nostoc* 584 was used as the template in the majority of the PCR reactions. The use of PCR to screen the phage library by the method of Cheng et al (1994) has also proved to be ineffectual (data not shown).

3.7 Screening of expression library in pTrc99A with Wsp antiserum

An expression library of *EcoRI*-*EcoRI* fragments of *Nostoc* 584 genomic DNA was generated and colony lifts from the plates were screened with the Wsp antiserum. One positive clone, VJ18, was identified on the basis of a strong cross-reaction with the antiserum. Plasmid DNA isolated from this clone was digested with *EcoRI* and the insert was found to contain three *EcoRI*-*EcoRI* fragments of the following sizes: 8.4 kb, 1.0 kb and 125 bp (data not shown). Each fragment was purified and cloned individually into the vector pTrc99A. Transformants from all three plates were screened again as above. Five transformants that contained the 8.4-kb *EcoRI*-*EcoRI* fragment, in the correct orientation
with respect to the trc promoter (pVJT4), were able to reproduce the original result of a
strong cross-reaction with Wsp antiserum. One of these cross-reactive clones, VJT4, was
analyzed further (see Fig. 10).

3.8 Sequence Analysis of pVJT4

The 8.4-kb insert in pVJT4 was sequenced on both strands. A partial restriction
map of the fragment is shown in Figure 10. Six open reading frames (ORFs) were
identified. The complete nucleotide sequence and the predicted amino acid sequences of the
six ORFs are shown in Figure 11. Each ORF is analyzed below

**ORF1**: Four ORFs can be identified which end at position 1922. The four
possible ATG start codons are located at positions 338, 387, 1182 and 1284 (see Fig. 12).
The polypeptide that is most likely to be expressed is the one that begins at position 387.
The putative ribosome-binding site for this ORF (hereafter referred to as ORF1), AGGA, is
7 nucleotides upstream of the start codon, position 376-379. ORF1 contains 516 amino
acids with a calculated molecular weight of 59,303. This corresponds well with the size of
one of the polypeptides overproduced by pVJT4 (see Fig. 13; section 3.8). The
polypeptide also exhibits a strong cross-reaction with the Wsp antiserum (see Figs. 12 and
13; section 3.8). A search of the Genbank database revealed highest sequence similarity of
ORF1 with glucosamine fructose-6-phosphate aminotransferase, an enzyme involved in the
formation of N-acetylglucosamine. Alignment of the two sequences shows a region of
similarity of 21 amino acids of which 11 residues are identical.

**ORF2**: This ORF contains 222 amino acid residues with the ATG start codon
located at position 2110 (see Fig. 12). The sequence AGGA (position 1997-2100),
precedes the start codon by 9 nucleotides, and is the consensus ribosome-binding site. The molecular weight determined from the deduced amino acid sequence is 24,340. This agrees well with the size of the second of the two overproduced polypeptides in pVJT4 (see Fig. 13; section 3.8). The amino terminus of this polypeptide has been determined and found to be an exact match with the deduced sequence (data not shown). There is no cross-reaction shown by this polypeptide against the Wsp antibody (see Figs. 12 and 13; section 3.8).

Comparison of the deduced amino acid of ORF2 with the Genbank database yielded highest similarity with pectate lyase (11 identical residues in a stretch of 21 amino acids) and dextran sucrase (13 identical residues in a stretch of 28 amino acids).

**ORF3**: The ATG start codon for this ORF is possibly located at position 2934 (see Fig. 12). There is no discernible ribosome-binding site for ORF3. The polypeptide starting at the Methionine residue would contain 200 amino acids with a molecular weight calculated at 22,352. Database searches have shown the highest sequence similarity of ORF3 with ExoR, an exopolysaccharide regulatory protein (9 identical residues in a stretch of 11 amino acids), Arbf, involved in utilization of β-glucoside (13 identical residues in a stretch of 34 amino acids), and a protein A precursor which binds peptidoglycan (12 identical residues in a stretch of 20 amino acids).

**ORF4**: There are two ATG codons (positions 3823 and 3889) present in this ORF. Neither of these codons is preceded by a consensus Shine-Dalgarno sequence. However, the sequence AGGA is located 13 nucleotides upstream of the GTG codon at position at 3697. Therefore, this GTG codon has been assigned as the putative start codon for ORF4. The encoded polypeptide contains 374 amino acid residues with a predicted molecular weight of 41,340. Comparison of the deduced amino acid sequence with the sequences in the databases suggested that ORF4 belongs to the family of proteins called
aldo/keto reductases. ORF4 contains one of the signature patterns listed for this group of proteins (Bairoch and Bucher, 1994; Accession # PS00062). The conserved pattern (see Fig. 4 legend) is as follows: [LIVMFY]-x(9)-[KREQ]-x-[LIVM]-G-[LIVM]-[SC]-N-[FY]. The sequence in ORF4 that corresponds to this motif, located in the central part of the protein, is L-D-A-V-V-R-A-G-K-V-R-Y-I-G-V-S-N-F. The best matches within this family are with 2,5-diketo-D-gluconic acid reductase (25 identical residues in a stretch of 48 amino acids) involved in the pathway leading to the synthesis of ascorbate, and xylose reductase (15 identical residues in a stretch of 23 amino acids) which reduces xylose to xylitol.

**ORF5**: There are multiple ATG codons in this reading frame but the most likely candidate for the translation start codon is the first one at position 5137. The reason for this choice is the presence of the sequence AGAGA, at a distance of 9 nucleotides upstream of the ATG codon, which could serve as a potential ribosome-binding site. ORF5 consists of 621 amino acid residues which corresponds to a molecular weight of 70,064. Database searches have identified ORF5 as belonging to the family of proteins known as ABC (ATP-Binding Cassette) transporters. This family of proteins has two signature patterns that are observed in all members of the family (Bairoch and Bucher, 1994; Accession # PS00017; Accession # PS00211). The consensus pattern for the ATP/GTP binding site motif (Accession # PS00017) listed in the PROSITE database is [AG]-x(4)-G-K-[ST] (see Fig. 4 legend). The sequence in ORF 5 that corresponds to the signature pattern is G-L-S-G-S-G-K-S, amino acid residues 395-402. The second signature sequence is as follows, Accession # PS00211: [LIVMFYC]-[SA]-[SAPGVYKQ]-G-[DENQMW]-[KRQAPCLW]-[KRNQSTAVM]-[KRACLVM]-[LIVMYPAN]-[PHY]-[LIVMFW]-[SAGCLIVP]-[FYWHP]-[KRHP]-[LIVMFYWSTA] (see Fig. 4 legend; residues listed within curly brackets '{ }' indicate that any residue is
acceptable at that position except the ones listed). In ORF 5 this motif is present as L - S - G - G - Q - R - Q - R - I - V - I - A - R - V - I at position 498-512. The highest similarities are seen with ChvA from Agrobacterium tumefaciens (Cangelosi et. al., 1989), NdVA from Rhizobium melilotii (Stanfield et. Al., 1988), and HetA (HepA) from Anabaena 7120 (Holland and Wolk, 1990). There is extensive similarity over a region spanning 224 amino acid residues which includes the putative ATP-binding site and the signature pattern for the family of ABC transporters (see Fig. 14). ORF 5 shows 47% identity with ChvA in this region; with conservative substitution of amino acid residues the similarity is 69%.

**ORF6**: A potential ORF consisting of 62 amino acids, starts at position 8228. ORF6 is not preceded by a consensus ribosome-binding site. The deduced amino acid sequence of ORF6 has a calculated molecular weight of 7,049 and shares sequence similarity with a small polypeptide from Arabidopsis thaliana known as cor6.6 (9 identical residues in a stretch of 17 amino acids).

### 3.9 Identification of the polypeptide that cross reacts with the Wsp antiserum

In the strains VJ18, VJT4 and VJT401 two proteins are overproduced upon induction with IPTG (Fig. 12(i), lanes F and H; Fig. 13(i), lanes C and E). The proteins have apparent molecular masses of approximately 59 kDa and 24 kDa. The sizes of these proteins correspond well with the predicted sizes of ORF1 and ORF2 respectively. These two ORFs are immediately downstream of the trc promoter in pVJT4 (see Fig. 10). The 59-kDa protein shows a strong cross reaction with the Wsp antiserum, the 24-kDa protein does not (Fig. 12(ii) lanes F and H; Fig. 13(ii) lanes C and E). Both the proteins are present in the subclone VJT401 but not in VJT403 (see Fig. 10; Fig. 13(i) lanes E and G).
This observation strengthens the probability that the 59- and 24-kDa proteins correspond to ORF1 and ORF2 respectively. The Western blots show the presence of additional bands that cross react intensely with the Wsp antiserum in the range 27 to 32 kDa (see Figs 12(ii) lanes F and H; 13(ii) lanes C and E). However, it can be seen from the CBB staining that these proteins are not overproduced (corresponding lanes in Figs. 12(i) and 13(i)). Attempts to overproduce ORFs 4 and 5 under the control of a T7 promoter have not been successful (see Fig. 13(i) lane F).
Discussion

The gene for fructose-1,6-bisphosphate aldolase, \( fba \), from \textit{Nostoc} 584 has been isolated and sequenced. The identification of \( fba \) is based on sequence similarities with class II aldolases from various organisms. The consensus patterns reported for class II aldolases are also present in the cyanobacterial \( fba \) (Bairoch and Bucher, 1994). \textit{Nostoc} 584 Fba exhibits remarkable similarity to CfxA and CfxB, two aldolases from the purple non-sulfur bacterium, \textit{Rhodobacter sphaeroides}. Cyanobacteria and purple bacteria are members of the phylogenetic group of photosynthetic prokaryotes (Doolittle, 1982). Both organisms are excellent models for examining biological processes such as fixation of carbon dioxide, photosynthesis and nitrogen fixation. Fructose-bisphosphate aldolase is a key enzyme in glycolysis as well as in the Calvin cycle for carbon dioxide fixation. It is likely that the aldolases in these organisms are specifically adapted to playing a role in both these metabolic activities. There is considerable correspondence of the sequence of \textit{Nostoc} Fba with those of the analogous enzyme from \textit{Bacillus subtilis}, (Fba1; Mitchell et al, 1992b; Trach et al, 1988) and the amino-terminus of Fba from \textit{Bacillus stearothermophilus} (Alefounder et al, 1989). The primary sequences of aldolases from \textit{Nostoc} 584 and \textit{B. subtilis} show 50% identity over the first 60 amino acids. However, the percentage of similarity decreases over the remainder of the sequence. The similarity of \textit{Nostoc} Fba to class II aldolases from \textit{E. coli} (Alefounder et al, 1989) and the yeasts, \textit{Saccharomyces cerevisiae} (Schwelberger et al, 1989) and \textit{Schizosaccharomyces pombe} (Mutoh and Hayashi, 1994) is discernible but less pronounced. The \textit{E. coli} and yeast aldolases are substantially similar whereas the \textit{R. sphaeroides} aldolases align well with the \textit{Bacillus}
proteins. As expected, *Nostoc* Fba and the other class II aldolases show no homology with the class I aldolases.

DNA sequence available at this time shows that *Nostoc* 584 fba is flanked on one side by *trnK*, the gene for lysyl-tRNA (see below). Information on the DNA sequence of the region adjacent to fba on the other side would shed light on its possible clustering with structural genes from either the glycolytic pathway or carbon dioxide fixation. The aldolase genes in *R. sphaeroides* are part of a cluster of structural genes of the pentose phosphate pathway (Gibson et al, 1991; Chen et al, 1991). CfxA is located in the form I carbon dioxide fixation operon whereas CfxB lies within the form II operon. The two operons have some structural genes in common (for example, ribulose-bisphosphate carboxylase) and have been mapped to distinct loci on the *R. sphaeroides* chromosome. The gene for aldolase, *fba*1 or *orfY*-tsr, (Trach et al, 1988; Mitchell et al, 1992b) in *B. subtilis* is downstream of the *spoOF* locus which encodes a protein required for sporulation. The gene product of *spoOF* belongs to a family of proteins that respond to environmental signals (Ronson et al, 1987). However, *fba*1 does not appear to be co-transcribed with *spoOF* which is thought to be monocistronic. The significance of the proximity of *fba*1 to *spoOF*, if any, is unclear. There is evidence to show that *fba*1 is co-transcribed with *orfU* in *B. subtilis* (Trach et al, 1988). A part of the deduced amino acid sequence of *orfU* corresponds to that of a transaldolase from *S. cerevisiae* but transaldolase activity has not been detected in *B. subtilis* (Mitchell et al, 1992a). The function of ORFU remains unknown. However, it has been demonstrated that Fbai and ORFU are phosphorylated during sporulation (Mitchell et al, 1992a). In *E. coli*, fba was isolated as a constituent of a cluster structural genes involved in glycolysis (Alefounder et al, 1989). In *Haemophilus influenzae* fba is adjacent to pgk, the gene encoding the enzyme phosphoglycerate kinase, which functions in glycolysis (Fleischmann et al, 1995). The two genes for class II
aldolases in *Alcaligenes eutrophus*, *cbbA*, are most distal to the promoter in both the *cbb* operons which include genes involved in the Calvin cycle of CO₂ fixation (Schaferjohann et al, 1995). Located downstream of *cbbG* and *cbbK*, the genes for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase respectively, *cbbA* has been grouped with the corresponding genes from *R. sphaeroides* and *B. subtilis*.

*Nostoc 584 fba* is transcribed from a promoter that is located between *trnK* and the ATG start codon of Fba. The putative -10 region of the *fba* promoter shows agreement with consensus sequences determined for cyanobacterial promoters; consensus sequences for the -35 region are poorly defined (Tandeau de Marsac and Houmard, 1987). In *Nostoc 584* the putative -10 and -35 regions of *rpoC1/C2* are TTAAAA and TTAGAA respectively (Xie and Potts, 1991). The putative -10 and -35 sites of the *Nostoc 584 fba* promoter match reasonably well with the consensus sequences that have been determined for *E. coli* promoters (-10 region, TATAAT; -35 region, TTGACA). The relatively low β-galactosidase activity (24.1 Miller units) measured suggests that the *fba* promoter may be a weak promoter. It should be taken into consideration that the assay was performed in an *E. coli* background. The *E. coli* and *Nostoc 584* RNA polymerases differ structurally, nevertheless, the RNA polymerases from the two organisms are similar enough for cross-recognition of promoters (Xie et al, 1989). Similar experiments performed to assess the promoter activity of the upstream region of *rpoC1/C2* in *Nostoc 584* (Xie and Potts, 1991) have yielded similar results; β-galactosidase activity was measured at 40 Miller units. In vitro experiments using the RNA polymerase from *Anabaena 7120* indicate that transcription is much more active from promoters that have sequences that resemble the consensus sequence for *E. coli* promoters as compared to transcription from strong *Anabaena* promoters (Schneider et al, 1991). The same study has also shown that
promoters of many genes expressed in vegetative cells of *Anabaena* 7120 are weakly transcribed by the major RNA polymerase. Results from in vivo experiments with the *Anabaena* 7120 RNA polymerase suggest that transcription from promoters similar to the *E. coli* consensus promoter is as high or higher than transcription from a strong *Anabaena* 7120 promoter (Elhai, 1993). Though the promoter-probe vector pCB267 can be used to test the relative strength of promoters, Schneider and Beck (1987) recommend caution in the interpretation of results from such comparisons since DNA sequences present in the 5' untranslated region between the promoter and the indicator gene can affect the expression of the latter. In the case of *Nostoc* 584 promoters, it may therefore be judicious to interpret the observed results qualitatively rather than quantitatively.

The ts8 mutation in PER1 is a single base change resulting in the replacement of a valine residue with glycine at position 300 in the amino acid sequence of *fba* in *E. coli* (Singer et al, 1991). In *C. glutamicum* and *S. cerevisiae* isoleucine is the residue corresponding to valine in *E. coli* (von der Osten et al, 1989). The valine (isoleucine) residue is located in a region of the sequence that is relatively well conserved in the three proteins. Therefore, there appears to be a requirement for a hydrophobic amino acid at that position. It has been well documented that the ts8 mutation is dominant to the wild-type (Singer et al, 1991). Overexpression of the *Nostoc* 584 *fba* would be necessary to overcome the dominant mutation in complementation experiments. We have been unable to clone the full-length *fba* for overexpression. The same fate was observed for *orfY-tsrt* (fbal) from *B. subtilis* (Mitchell et al, 1992b). The authors hypothesized that this may have been a consequence of that region of DNA being toxic to the host cell in some unknown manner. In this study two truncated forms of the *Nostoc* 584 *fba* have been expressed in *E. coli* (Section 3.4). Of the two, only the longer polypeptide (encoded by pVJA52),
containing the first 291 amino acids, is present in sufficient amounts to counteract the
dominance of the ts8 mutation. However, it was observed that pVJA52 does not
complement the ts8 mutation in PER1 (see Fig. 7). It is likely that the shorter form of the
polypeptide is not sufficient for complementation of the ts8 mutation even though it
contains both signature patterns for class II aldolases. The 68 amino acid residues that are
missing at the carboxy-terminal end in the truncated Nostoc 584 aldolase may be necessary
for the activity of the enzyme. Therefore, a longer version of the aldolase, preferably full-
length, would probably be able to complement the mutation. However, the lack of
complementation could potentially be due to the specific requirements of the Nostoc 584
aldolase, such as cofactors or processing, which cannot be met by E. coli. So, it is
conceivable that overexpression of the complete Nostoc 584 fba may not complement the
mutation. The R. sphaeroides aldolase could not be assayed when overproduced in E. coli
(Gibson et al, 1991). Since the Nostoc 584 Fba shares much more homology with the R.
sphaeroides enzyme, as compared to E. coli, it is very likely that the Nostoc 584 enzyme
too would be inactive in an E. coli background. Wild type and mutant strains of E. coli
transformed with pVJA52 show a drop in aldolase activity upon induction (see Fig. 7).
Since class II aldolases behave as dimers (Baldwin et al, 1978), this could be due to the
phenomenon known as subunit poisoning.

The deduced amino acid sequence of Fba from Nostoc 584 is the first report of the
primary sequence of a class II aldolase from cyanobacteria. In Nostoc 584 fba was isolated
from an expression library using the Wsp antiserum. The truncated forms of fba expressed
in E. coli do not cross react with the Wsp antibody (data not shown). The 1.1-kb EcoRI-
EcoRI fragment isolated from the λgt11 library may have been the result of a false positive
reaction. However, the isolation of fba from Nostoc 584 will allow further investigation of
hexose metabolism and the coordination of overall carbohydrate metabolism in this organism. This information could provide insights into the synthesis and regulation of EPS which is essential to the understanding of the mechanism of desiccation tolerance in *Nostoc 584*. The availability of *fba* will also permit genetic manipulations to study the relationship between mutations in this gene and the synthesis of stable RNA species.

In *Nostoc 584*, *fba* is located downstream of the gene encoding lysyl-tRNA (*trnK*). In *E. coli*, tRNA genes are generally clustered and may be present in the form of operons. Genes coding for tRNAs are also found as part of rRNA operons (*rrnA-G*). From nucleotide sequence available at the present time it is not possible to determine whether the *Nostoc 584 trnK* is part of a cluster of tRNA genes or an *rrn* operon. This analysis will have to await isolation and sequencing of the region flanking *trnK* at its 5’ end.

The recombinant plasmid pVJ004 contains a 1.1-kb EcoRI-EcoRI insert (see Fig. 1). The insert contains *trnK* and approximately half of *fba*. It was observed that pVJ004 could not be maintained stably in a variety of *E. coli* strains (see Section 3.2). Transformation of these strains with pVJ004 was successful. The transformed cells were viable and could be selected for by incorporating ampicillin in the medium. However, a few days after transformation it was no longer possible to isolate plasmid DNA from these strains. The reason for the instability of pVJ004 is unclear. A search of the Genbank database using the nucleotide sequence of the 1.1-kb fragment from pVJ004 revealed similarity between *trnK* and immediately flanking region with a portion of the genomes from the bacteriophages HP1 and HP1c1. This region of bacteriophage DNA included the site involved in site-specific integration of the phage genome into the chromosome of its host *Haemophilus influenzae* (Waldman et al, 1987; Goodman and Scocca, 1989). These lysogenic phages insert the prophage genome at a single, specific site (*attB* in the host and *attP* in the phage) in the host chromosome using a recombination reaction. The phages
target tRNA genes for insertion (Reiter et al, 1989). The att sites, in host and phage contain almost identical regions that are 183 bp in length. This region can be split up into 3 parts: a 93-bp region of perfect identity; a 27-bp region with 6 mismatches and a 63-bp region of perfect homology. Contained within the 93-bp region is a complete sequence of the gene for lysyl-tRNA. Deletion clones containing chimera that did not carry the trnK segment of pVJ004 were found to be maintained stably.

Exhaustive attempts to isolate wsp using the technique of PCR have been unproductive. The ineffectiveness of the PCR approach may be due to a number of reasons. Firstly, the choice of oligonucleotides used in the PCR experiments was restricted by the availability of limited amounts of amino acid sequence data (Hill et al, 1994a). Also, it has been observed that certain pairs of oligonucleotides are incompatible (Innis and Gelfand, 1990). The formation of primer dimers may be one reason for the incompatibility.

Peptide sequencing of additional proteolytic fragments of Wsp will provide information that can be used to design more oligonucleotides. Secondly, the sequences of the oligonucleotides synthesized were based on the portions of the amino-terminal and internal peptide sequences of Wsp from field material of N. commune (Compton, 1990). Little is known about codon usage in the field material of N. commune which, as the term “field material” suggests, has been collected from its natural habitat and is very different from Nostoc 584 which is a clonal axenic strain that has been well characterized. Therefore, the oligonucleotides were synthesized either as mixtures or with inosine in the wobble position to account for redundancy of the genetic code (see Fig. 9). In a mixture it is assumed that the components of the mixture are present in stoichiometric amounts. This assumption may not necessarily be true and could be a cause of the lack of success observed in PCR experiments. The inosine-containing oligonucleotides partially overcome the limitations of working with the mixtures.
Genomic DNA isolated from field material of *N. commune* was not used as a template in PCR reactions because of the presence of contaminants in the field material. Recently, after much effort, one strain of the field material (DRH1) was purified in our laboratory (Hill, unpublished results). Isolation of *wsp* by the PCR approach may now be possible by employing genomic DNA from the strain DRH1 as the template.

A search of the Genbank and Swiss-Prot databases has revealed that ORFs 1 through 6 exhibit some similarities to known proteins. However, it is not possible at this time to ascertain the identities of these six ORFs. ORF6 shows highest similarity with a protein of comparable size from *Arabidopsis thaliana*. The protein known as Cor6.6 is induced by abscissic acid, cold, and, like *Wsp*, water stress. Cor6.6 is almost identical to the protein Kin1, also from *A. thaliana*, and shows sequence similarities with the anti-freeze proteins found in fish (Gilmour et al., 1992). The product of ORF1 has an apparent molecular mass of 59 kDa. It has been shown in Western analysis of total cell protein extracts of *Nostoc* 584 that there is a band that cross reacts with the *Wsp* antibody and is similar in size to the product of ORF1 (D.R. Hill, personal communication). The identity of this cross reactive band is unknown at this time. However, it is possible that this band may represent the expression of ORF1 *in vivo*. The predicted amino acid sequences of the six ORFs do not match the amino-terminal or internal sequences determined for the *Wsp* polypeptides (Hill et al., 1994a). Nonetheless, it is abundantly evident from Table 3 that ORFs 1 - 5 share the common feature of carbohydrate metabolism/synthesis. It is postulated that ORFs 1 - 5 represent novel genes involved in carbohydrate metabolism and that these genes constitute all or part of an operon. The probable roles played by the genes in this operon in the synthesis, secretion and/or modification of the glycan sheath and its components, *Wsp* and the UV-A/B-absorbing pigment are discussed below.
Synthesis of polysaccharides is one of the ways by which bacteria respond to desiccation and it is believed that EPS play a protective role during drying (Potts, 1994). The disaccharide trehalose has been shown to stabilize membranes, proteins and whole cells (Carpenter and Crowe, 1989; Crowe et al, 1987; ). The interaction between trehalose and phospholipid bilayers in the dry state is thought to occur by the disaccharide replacing the water molecules around the polar head groups of the membranes ("water replacement hypothesis"; Clegg, 1986). The disaccharide sucrose is known to stabilize membranes in dry cells (Hoekstra et al, 1991; Hoekstra et al, 1992). Carbohydrate metabolism and Wsp appear to be tightly linked in *Nostoc commune*. Wsp antiserum is known to cross react with the enzymes N-glycosidase F and β-D-galactoside galactohydrolase. Wsp is thought to be involved in events leading to either the synthesis, the secretion, the binding, the modification and/or the turnover of oligosaccharides in *Nostoc commune* (Hill et al, 1994b). Field material of *N. commune* secretes abundant amounts of glycan which accumulate extracellularly (Hill et al, 1994a). This glycan sheath forms a protective barrier between the filaments of *N. commune* and its environment and may play a role in protecting cells during water deficit (Hill et al, 1994b). Wsp are secreted into this glycan sheath along with the UV-A/B-absorbing pigments that protect the cells from long-term UV exposure. The amino-terminal sequence of Wsp shows correspondence with carbohydrate-metabolising enzymes (Hill et al, 1994a). The sequence showing the highest similarity is that of a N-acetyl-D-glucosamine-binding lectin/chitinase. Formation of N-acetyl glucosamine is the first step in chitin (poly N-acetyl glucosamine)biosynthesis. ORF1, which exhibits a strong cross reaction with Wsp antiserum, shows similarity with an enzyme required in the formation of N-acetyl glucosamine. Database searches have shown that the internal sequence of Wsp corresponds to portions of a β-xylosidase. Wsp extracts
demonstrate a weak xylosidase activity but a xylanase activity is associated with Wsp extracts from the extracellular sheath (Hill et al, 1994a). Though xylose is not detectable as a component of the glycan sheath it is a constituent of the polysaccharide core of the UV-A/B-absorbing pigment in *N. commune* (Bohm, 1993). The deduced amino acid sequence of ORF4 shows similarities to aldo-keto reductases such as xylose reductase and 2,5-diketo D-gluconic acid reductase. The latter is involved in the pathway leading to ascorbate synthesis. The chromophore of the UV-A/B-absorbing pigment has a structure remarkably similar to ascorbate (an ene-diol). Glucuronic acid and galacturonic acid (see ORF2) are used to synthesize ascorbic acid in plants. Uronic acids have been found to be present in the EPS of bacterial cells (Hill et al, 1994b).

The amino acid sequence of ORF5 shows that it belongs to the family of proteins known as ABC (ATP-Binding Cassette) transporters. A majority of proteins which bind the nucleotides ATP or GTP have a number of sequence features in common. The sequence pattern that is most conserved is called the ‘P-loop’ (Saraste et al, 1990) or the ‘A’ consensus sequence (Walker et al, 1982). The motif consists of a region rich in glycine residues which is thought to form a flexible loop that interacts with a phosphate group of the nucleotide. Within the family of ABC transporters, ORF5 shows highest similarity with ChvA from *Agrobacterium tumefaciens*, NdVA from *Rhizobium meliloti*, and HetA (HepA) from *Anabaena* 7120. ORF5 shows 47% identity at the amino acid level with ChvA over a region spanning 224 amino acids. This region of identity includes the ATP-binding site and the signature pattern for ABC transporters. Both ChvA and NdVA are transport proteins involved in the export of cyclic glucans. These polysaccharides are synthesized exclusively by the members of the family *Rhizobiaceae* and play a role in osmoregulation under hypoosmotic conditions (Sutherland, 1985; Miller et al, 1986). The periplasmic cyclic glucans are also required for attachment of the bacteria to plant cells (Breedveld and
Miller, 1994). HetA (HepA) is involved in the accumulation of the polysaccharide envelope layer of heterocysts in the cyanobacterium *Anabaena* (Holland and Wolk, 1990). It has been observed that Wsp antibodies collect at the periphery of the envelope layer of heterocysts of *Nostoc 584* (Hill et al, 1994b). ORF3 exhibits highest sequence similarity with ExoR which is a negative regulator of exopolysaccharide production in *Rhizobium meliloti*. Mutations in exoR cause overproduction of an exopolysaccharide called succinoglycan and result in the inability of the mutant to invade the root nodules of the host plant (Reed et al, 1991). ORF3 also shows similarity to ArbF which is a component of the phosphoenol-pyruvate-dependent sugar phosphotransferase system (PTS), a major active transport system for carbohydrates (El Hassouni et al, 1992).

Synthesis and secretion of the glycan sheath and its components represents a substantial investment of the carbon resources of *N. commune* and would require coordinated regulation of a number of genes involved in carbohydrate metabolism. A better understanding of the identities and functions of ORFs 1 through 5 may yield clues to their relationship to Wsp and their possible role in desiccation tolerance.
**Table 1. Plasmids and Chimeras used in this study**

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<td>-</td>
<td>Promega</td>
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<tr>
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</tr>
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<td>This study; Fig. 5</td>
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<td>This study; Section 3.7</td>
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</tr>
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<td>This study; Fig. 1</td>
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<td>This study; Fig 10</td>
</tr>
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<td>pVJT403</td>
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<td>This study; Fig 10</td>
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49
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<tr>
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<tr>
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<td>DH10B</td>
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<td>1</td>
<td>Glucosamine fructose-6-phosphate-aminotransferase</td>
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Figure 1. Restriction map of the region of *Nostoc* 584 DNA containing *fda*.

Rectangles represent genes with the following designations: *fda*, fructose-1,6-bisphosphate aldolase; *trnK*, lysyl-tRNA. The arrows indicate direction of transcription. The heavy bars above and below the map denote plasmid constructs. The light bars indicate the probes used in hybridization experiments. The dashed line shows the portion of the DNA fragment that has been sequenced. Restriction endonucleases are: E, *EcoRI*; P, *PstI*; H, *HindIII*.
Figure 2. Nucleotide sequence and deduced amino acid sequence of *Nostoc* 584 *fda* and flanking region. The predicted amino acid residues are represented by the one-letter code. The putative ribosome-binding site is underlined. Potential -10 and -35 sites of the promoter region are indicated by dotted underlining. The translation stop codon is marked by a dash (−). The gene for lysyl-tRNA, *trnK*, has been double underlined. *EcoRI* and *HindIII* sites are shown in italics.
Figure 3. Sequence alignment of the deduced amino acid sequence of *Nostoc* 584 Fda with two fructose bisphosphate aldolases from *Rhodobacter sphaeroides*, CfxA and CfxB. Identical residues are indicated by a line (1) and residues in the same group (ILVM, YFW, HKR, QNED, PAGST) are indicated by a colon (:). Signature patterns for class II fructose-bisphosphate aldolases are marked with an asterisk (*).
Consensus pattern #1 : Accession # PS00602


Y P Q I P I C L H Q D H G

Consensus pattern #2 : Accession # PS00806

[LIVMF]-X(2)-[GSA]-[LIVM]-X(2)-G-X(2)-K-[LIVM]-N-[LIVM]-[DN]-T-[DE]

I E R G I R H G V R K V N I D T D

Figure 4. Fructose-1,6-bisphosphate aldolase (class II) signature patterns.

Each pattern entry in the PROSITE database has a unique accession number. Amino acid residues in the pattern are represented by the one-letter code. The letter ‘X’ denotes that any amino acid is acceptable in this position; X(2) indicates that any two residues are acceptable; X(1,3) means that a range of one, two or three amino acids are permissible. Residues enclosed within square parentheses, [ ], are a list of acceptable amino acids for that position. Below each signature pattern is given the actual amino acid sequence from Nostoc 584 Fda that corresponds to the pattern.
Figure 5. Cloning strategy to assay for promoter activity. The restriction map shown here is that of the 1.1-kb insert from the plasmid construct pVJ004 (see Fig. 1). The restriction sites on the map are F, *EcoRI* and S, *Sau3A*I. The bar below the map denotes the DNA fragment that was cloned into the promoter probe vector pCB267 to yield the chimeric plasmid pVJ008.
Figure 6. **Identification of promoter region of fba**. The assay was performed as described in Section 2.11. The parent *E. coli* strain in both samples is TL73 (ΔlacZ). The *E. coli* strain VJ008T contains the chimeric plasmid pVJ008 (see Fig. 5). The strain CB267T contains the promoter probe vector pCB267. For description of units see section 2.11.
Figure 7. Activity of truncated *Nostoc 584 fba* in *E. coli*. The assay was performed as described in section 2.12. All the strains have been induced with IPTG. Wild-type activity was taken to be 100% and all other activities are represented as a percentage of wild-type activity. Strains PER4 and PER1 are the wild-type and the *fda* mutant respectively. Strains PER4-A52 and PER1-A52 are as above except that they have been transformed with the chimeric plasmid, pVJA52 (see Fig. 1), that overproduces a truncated form of *Nostoc 584 Fda*. For description of units see section 2.12.
Figure 8. Sequence alignment of the lysyl-tRNA genes (trnK) from chloroplast of liverwort, Marchantia polymorpha (M.p.) and Nostoc 584 (N.c.). Identical nucleotides are indicated by an asterisk (*). The anticodon, TTT has been double underlined.
Figure 9. Oligonucleotides synthesized for PCR. Mixtures of oligonucleotides were synthesized based on the partial amino acid sequence of Wsp. Parentheses indicate the nucleotides possible at that position. I; represents nucleotides containing inosine. Restriction sites engineered within the oligonucleotides are shown in italics. The oligonucleotides, #97015 and #97044 are based on the amino-terminal sequence determined for Wsp, YGYTIGE; #39224 and #39235 are based on the internal amino acid sequence of Wsp, EARVTGP.
# 97015 : 30-mer

**EcoRI**


# 97044 : 30-mer

**EcoRI**

5' C T C A G A A T T C T A (C/T) G G I T A (C/T) A C I A T I G G I G A 3'

# 39224 : 30-mer

**HindIII**

5' T A C T A A G C T T G G (A/C/G/T) C C (A/C/G/T) G T (A/C/G/T) A C (A/C/G/T) C (G/T) (A/C/G/T) G C (C/T) T C 3'

# 39235 : 30-mer

**HindIII**

5' T A C T A A G C T T G G I C C I G T I A C I C (G/T) I G C (C/T) T C 3'
Figure 10. Restriction map of the 8.4-kb EcoRI-EcoRI fragment isolated from the expression library of Nostoc 584 genomic DNA in the plasmid vector pTrc99A. The 8.4-kb DNA fragment is from the chimeric plasmid pVJT4. The restriction sites are: E, EcoRI; S, SalI. The rectangles represent open reading frames (ORFs). The light arrows show the direction of transcription. P_{trc}; the trc promoter with direction of transcription indicated. The shaded ORFs are the ones that are overproduced in strain VJT4 upon induction with IPTG. The heavy bars indicate the inserts in subclones, VJT401 and VJT403.
Figure 11. **Nucleotide sequence and deduced amino acid sequence of the 8.4-kb insert in pVJT4.** The predicted amino acid residues are represented by the one-letter code. The putative ribosome-binding sites are underlined. The translation stop codon is indicated by a dash (-). *EcoRI* and *SalI* sites are shown in italics.
EcoRI
GATTCGTTAACCTAGTGGTTCCGCCTCTTCACAGAGGGACTGTTGGGCTAGCAATAC
GAAAGGCAGTACTTGGTGAAAACCTCCTGGGTGTGCTGGCaATGCTTTTGCGCCTGA
AAACAACTAGTGAAGATGGTTTGGCGTAAATGACTTTTCATATCTGACTGTGCTT
ATAGTGCTGTACGGTAAATGAAATTTTTCAGGGATGATTAAAAGCATACGCTTAAATTT
CCCTCTGTAGGATCGGCCTCAGAAACTGAAATGTTGGAAGAGCTGTAATATGGCC
GTCTCTCACAAAAATCTATGCGTAATAGCAACATTGGATGCTAGGAAAGAAACCTGTACTT

ORF 1
TTTGTGCGTGAGATAAGGAADAAAAACATGACTGAAATATAGGCGATTGAAAGAACCTCAGCT
MTEYSRLKTSR

CTGCTGCAATCAGAGCAACCTTGATTATCGGTAAATCGACCTGACCTGTTACACCAATG
SAAIRATLDDYPVIDTDVHTN

ATTCAAGCCGACTTTTGGAGATTACATCTGCTAAGTACCCGGGCGGTTGAGAAACTGTGATG
DFTPAFEDYIAKYGGVKLVD

AGTTAACGTAAAGGGAAGCGCTTTGGCGTCTAACCACCGAAAGGCTAAAGACTCGTTATC
ELRKTSEASRLNSKSNKGDYW

AAACACGGGCTGAAAGGGCTAATACACGCGGACATCAGGATCGCTGTTGGGCTAGAG
QQTPEERQYNRITRSPWWR

TTACCCAAAAACACATTGGATCTCGCTACTTACACCCCTCGCGGACTCTCTATGAGGTC
VTKnLDLATYTLPGLLYER

AGGCGGAAACAAAGGATCGGAACATTCGCGCTGTTCCACAAATGTTCTAGCGAAGCGCG
QAEGQGSDYSVLFPNNVLAPA

GAGCAGTGCGAGAAATGCTCAAGCCATTACAACCGCGCAGTCATCAGTATCTACGCGTAAG
GASNEQRALQRARNYHAD

TCTACGGAAATATAGGCGGCTTGAGACCCGCTCTGCTGCTATTAGCTACTACTCGG
IYRKYSDRLTPVAGIPTTTP

AAGAGGGCATGAGGACATACGTTTGGCTGAAAACACTGGCGCTTAAAGGTAATATATA
QEGIEELEFAVKTGLKVIN

TTACCGGGGCCTGTTGAAAGCGGGCAATCAAAAGCGATGCTGTAAATATCGAAGCTGTAAT
ITGGVKRPKIAIDKYPADK

ACCCGGAAATATCGGTAATGCTCTTATATGCGACTTCTACGGGATAGAAGATGGAATCG
YPEIAYKSYIDFYGLDSEY

70
AGAGGAGATTTCTCTCATTAGAAATTAGAAATAGGAAAATGCGAATGACACATGACCA
G  R  D  S  L  R  
4860
ATGACCAATAGCTCAATACGAAATATATATGCTAGAAAATACTGATGATTAAACGGCCGT
4920
CGTTTCTCTCTATTGCTCTTCTCCGACTATGCTGTTTCTATGCGATTATCCTGTATAGTA
4980
AGGTGTAGGGAAACAAAACACAAATAATTCTGCAATCAACACAAACAAATTTGATGCTAA
5040
AGTACTCTTAAAGTGCTCTATTGCTGAAAAGGCTGATTGCTGTGGATTGTTAAGTCCTATT
5100
ORF 5
5160
MTKKARQ5
5220
AAATCCTTTTCAACGGTCTCTCTGCTGACACCAGATTTACCCATACCTTCAATTTACTAGGTTCTAT
KFQQRAPDAPDLPSTPFRFI
5280
TTGTATTCTGTTGAAACAGAGTTCCTGTGCTATTGTTATGTTAAATGATGCTGTGGATTGAAATA
LYFVNVQFRRWYWYVLMVILEVI
5340
CAACGCAACTGTTGCGATCTGTTCTTATGCTATTTGTTGAAATCATTTTGGGCGCTGGTCG
HATCGIMLPYAIAGEIIIRGV
5400
CGATGACGCGGGGGCAACCAAACCTATTCTTTTGGATGAAATCCTGTGATGATTTCTTCT
RSTGDNKIFDAMKQPLMLF
5460
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IGLGSVGEVVFGRSAGGLLQTI
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YSRKAAAAARSETTGTGIVDTV
TGGACTACCCAGAATGAGTTTTTAGACAGAAAAACAGCCTAGAAAAACCTATGGCAATT
GLPE

EcoRI
ATACTACATTGCCCAGAATT
Figure 12. Identification of overproduced polypeptides in total cell protein extracts. (i). CBB stained SDS-PAGE gels. (ii). Corresponding Western blots using Wsp antiserum. Uninduced samples are marked with a minus sign (-); samples induced with IPTG are indicated with a plus sign (+). Total cell protein extracts in lanes A and B are from the strain DH10B; lanes C and D are from strain DH10B transformed with the plasmid vector pTrc99A; lanes E and F are from strain VJ18; and lanes G and H from strain VJT4. Lane I contains Wsp polypeptides purified from rehydration fluids of field material of Nostoc commune. Positions of prestained molecular weight markers (in kDa) are indicated by arrowheads.
Figure 13. Identification of overproduced polypeptides in strain VJT4 and its subclones. (i) CBB stained SDS-PAGE gels. (ii) Corresponding Western blots using Wsp antiserum. Uninduced samples are marked with a minus sign (-); samples induced with IPTG are indicated with a plus sign (+). Lanes A and H contain Wsp polypeptides purified from rehydration fluids of field material of *Nostoc commune*. Total cell protein extracts in lanes B and C are from the strain VJT4; lanes D and E are from VJT401; and lanes F and G are from VJT403. Positions of prestained molecular weight markers (in kDa) are indicated by arrowheads.
Figure 14. Alignment of part of the deduced amino acid sequence of ORF5 from *Nostoc 584* (N.c.) with that of ChvA from *Agrobacterium tumefaciens* (A.t.). Residues 324 to 547 from N.c. have been aligned with residues 333 to 556 from A.t. Identical residues are marked with a line (l) and residues in the same group (ILVM, YFW, HKR, QNED, PAGST) are marked with a colon (:). The consensus sequence for the ATP/GTP-binding site is indicated by a broken double line (==). The signature pattern for ABC transporters is marked with asterisks (*).
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Appendix

Composition of buffers and media used in this study

1. TAE Buffer (50-fold strength)
   Per liter:
   Tris base 242 g l⁻¹
   glacial acetic acid 57.1 ml
   0.5 M EDTA (pH 8.0) 100 ml

2. TBS (5-fold strength)
   Per liter:
   Trizma base 12.1 g
   NaCl 146.2 g

   Dissolve the solids in 800 ml water, adjust pH to 7.5 and bring volume to 1 liter with distilled water.

3. TBST
   Per liter:
   5-fold strength TBS 200 ml
   Tween 20 500 µl

   Bring volume to 1 liter with distilled water.
4. CAPS Buffer

Per two liters:

\[
\text{CAPS} \quad 4.425 \text{ g}
\]

Degas thoroughly. Prior to use add 220 ml 100% methanol and adjust pH to 10.3 with 1N NaOH

5. TE Buffer (pH 7.5)

10 mM Tris.HCl (pH 7.5)
1 mM EDTA (pH 8.0)

6. Gel Loading buffer II (10-fold strength)

0.25% bromophenol blue
0.25% xylene cyanol
25% Ficoll (type 400) in \( \text{H}_2\text{O} \)

7. LB (Luria-Bertani) Medium

Per liter:

\[
\begin{align*}
\text{Bacto-tryptone} & \quad 10 \text{ g} \\
\text{Bacto-yeast extract} & \quad 5 \text{ g} \\
\text{Sodium chloride} & \quad 10 \text{ g}
\end{align*}
\]

Adjust pH to 7.5 with sodium hydioxide
8. LB Agar or Agarose

Make up liquid medium as described above. Prior to autoclaving add (per liter)

Agar 15 g (for plates)
Agar 7 g (for top agar)
Agarose 7 g (for top agarose)

9. M9 Minimal Medium

Per liter:

5-fold strength M9 salts 200 ml
(see below)

20% Glucose 20 ml
1M MgSO₄ 2 ml
1M CaCl₂ 0.1 ml.

5-fold strength M9 salts; per liter

Na₂HPO₄·7H₂O 64 g
KH₂PO₄ 15 g
NaCl 2.5 g
NH₄Cl 5.0 g
Vita

Vinita Joardar (nee Babulkar) was born in Visakapatnam, India. She completed the tenth grade at Lawrence School, Lovedale and graduated in the twelfth grade, in 1980, from St. Xavier's College, Bombay. In 1983, she obtained a Bachelor’s degree in Microbiology from the University of Bombay. Continuing at the same university she received a Master’s degree in Biochemistry, in 1985. She worked as a Junior Research Fellow at the Institute of Science until 1988. She joined the graduate program in the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute and State University in 1989, and worked under the guidance of Dr. Malcolm Potts. She defended her doctoral dissertation in September, 1995.

She is married to Saikat Joardar. They currently reside in Pensacola, Florida, with their daughter, Antara Rohi.