

A transposition-induced mutant of *Nostoc ellipsosporum* implicates an arginine-biosynthetic gene in the formation of cyanophycin granules and of functional heterocysts and akinetes

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In strain NE1 of Tn5-1058-mutagenized *Nostoc ellipsosporum*, the transposon was found within a gene whose translation product is similar in amino acid sequence to the arginine-biosynthetic protein *N*-acetylglutamate semialdehyde dehydrogenase encoded by *argC* of *Bacillus subtilis*. The *argC* reported from *Anabaena* sp. strain PCC 7120 hybridized to a sequence different from the one interrupted by the transposon in NE1. The newly identified gene from *N. ellipsosporum* was denoted *argL*. The *argL* mutation renders certain processes in strain NE1 conditionally dependent on provision of L-arginine. Heterocysts and apparent akinetes that formed in the absence of added L-arginine failed to fix dinitrogen or to germinate, respectively, and lacked granules of cyanophycin, composed of copolymers of arginine and aspartic acid. However, apparent akinetes that differentiated upon growth of the mutant in the presence of L-arginine plus nitrate formed cyanophycin granules and could regenerate a new culture.

Keywords: akinete, arginine, cyanophycin, differentiation, heterocyst

INTRODUCTION

When certain filamentous cyanobacteria are grown in the presence of fixed nitrogen, all of the cells have similar morphology, and are known as vegetative cells. When the filaments are deprived of nitrogen, vegetative cells at semiregular intervals along the filaments differentiate into nitrogen-fixing heterocysts (Buikema & Haselkorn, 1993; Wolk *et al.*, 1994). Different types of environmental stress (Sutherland *et al.*, 1979; Wolk, 1965) lead to the formation of another kind of specialized cell, the akinete or spore.

Several observations suggest that the alternative differentiation processes may be related. In filaments of some organisms, akinetes arise where heterocyst formation might have been expected (Geitler, 1925). In *Anabaena* species, the envelopes of heterocysts and akinetes contain equivalent polysaccharides (Cardemil & Wolk,

1976, 1979, 1981). Soriente *et al.* (1993) reported that akinetes contain glycolipids characteristic of heterocyst envelopes (Winkenbach *et al.*, 1972). The further suggestion has been made (Wolk *et al.*, 1994) that heterocysts may be evolutionary derivatives of akinetes. Evidence has been presented that genes *hetR* and *hepA* are involved in the differentiation of both heterocysts and akinetes (Leganés *et al.*, 1994; Leganés, 1994). To pursue analysis of the differentiation of akinetes, and to test further the presumptive relationship between the differentiation of heterocysts and akinetes, we made use of *Nostoc ellipsosporum*, a genetically manipulable cyanobacterium that can form both these types of cells (Leganés *et al.*, 1994).

Cyanophycin is a nitrogenous reserve material, easily visualized in the form of refractile granules by phase-contrast microscopy, that normally consists of copolymers of arginine and aspartic acid (Simon, 1971; see, however, Merritt *et al.*, 1994). It is often present in the polar regions of heterocysts (Lang *et al.*, 1972) and is frequently abundant in akinetes (Herdman, 1987). Here, we report analysis of transposon mutant NE1 of

Abbreviation: DW, dry weight.

The GenBank accession number for the sequence reported in this paper is U48355.

N. ellipsoforum, whose akinetes and heterocysts are morphologically abnormal and, as is true also of the vegetative cells of the mutant, lack cyanophycin granules in the absence of exogenous L-arginine. The lack of these granules correlates with impaired functionality of both types of differentiated cells.

METHODS

Strains, plasmids and culture conditions. Axenic cultures of *N. ellipsoforum* derived from strain B1453-7 of the algal culture collection of the Plant Physiology Institute of the University of Göttingen, and strains of *Escherichia coli*, were grown in liquid media or on agar-solidified media as previously described (Hu *et al.*, 1981; Wolk *et al.*, 1984). AA/8 is one-eighth strength AA medium. Akinetes were formed in the presence of standard sporulation medium supplemented, when specified, with 25 mM calcium glucuronate obtained from Mann Research Laboratories (Wolk, 1965). The concentrations of antibiotics used in the initial selection for transposon-bearing exconjugants, upon transfer of pRL1058 to *N. ellipsoforum* (Leganés *et al.*, 1994), were 200 µg neomycin sulfate ml⁻¹ and 10 µg streptomycin sulfate ml⁻¹. Suspension cultures of mutant NE1 were supplemented with 40 µg neomycin sulfate ml⁻¹ and 5 µg streptomycin sulfate ml⁻¹. Cultures of strain DR1505 (see below) were supplemented with 2.5 µg streptomycin sulfate ml⁻¹ and 2.5 µg spectinomycin ml⁻¹. Where indicated, cultures were supplemented with 1 or 4 mM L-arginine hydrochloride (Sigma).

Analytical methods. For dry weight (DW) determinations, cells were collected and dried at 70 °C for 48 h. Nitrogenase activity was determined by the acetylene reduction method (Stewart *et al.*, 1967). Cyanophycin granules were isolated by the method of Allen (1988), which is based on that of Simon (1971). Isolated cyanophycin granules were treated with 0.1 M HCl, and the arginine from these granules was measured by the modified Sakaguchi reaction of Messineo (1966).

Recovery of DNA contiguous with the transposon, reconstruction of the mutation, and Southern analysis. DNA contiguous with Tn5-1058 was recovered from the genomic DNA of cyanobacterial mutant NE1 by excision with *EcoRV*, circularization and transfer to *E. coli* by electroporation (Wolk *et al.*, 1991), producing plasmid pRL1429. In an effort to reconstruct the original mutation that is present in NE1, pRL1429, cut with *PstI* and *BamHI* to eliminate most of the transposon, was ligated to pRL759D (Black *et al.*, 1993) that had been cut with *PstI* and *BamHI*, producing plasmid pRL1441D. Plasmid pRL271 (Black *et al.*, 1993) was cut with *NruI* and *ScaI*, and the large fragment, containing *sacB* and determinants for resistance to chloramphenicol and erythromycin, was introduced into the *EcoRV* site of pRL1441D, producing pRL1501. Cassette C.S4, present in plasmid pRL65 (Fernández-Piñas *et al.*, 1994), confers resistance to streptomycin and spectinomycin. The *lux*-bearing fragment of pRL1501 bounded by sites for *BamHI* and *BglII* was replaced by cassette C.S4 (bracketed by *BglII* sites) from pRL65, producing pRL1505. This plasmid was introduced into wild-type *N. ellipsoforum* by conjugation (Elhai & Wolk, 1988). Double recombinant strains were selected as described by Cai & Wolk (1990) on AA medium with nitrate (Allen & Arnon, 1955), streptomycin, spectinomycin, 5% sucrose and 1 mM L-arginine. For Southern analysis of chromosomal DNA from these strains, DNA probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim) from random primers (Gibco-BRL).

Sequence analysis. Automated sequencing (Applied Biosystems) was performed on restriction fragments that were subcloned from pRL1429 to pUC18 and pUC119 (Yanisch-Perron *et al.*, 1985; Vieira & Messing, 1987). Both strands of DNA were sequenced; database comparisons and alignments of the DNA and of predicted sequences were performed with the default settings of the algorithm developed by Altschul *et al.* (1990) using the network service programs BLASTN and BLASTP of the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Phenotype of mutant NE1

Transposon-generated mutants of *N. ellipsoforum* affected in the development of heterocysts were identified by their inability to assimilate N₂ (Ernst *et al.*, 1992). When one such mutant, strain NE1, was transferred to medium without combined nitrogen, the doubling time, estimated from the increase in dry weight, of the mutant was significantly greater than that of the wild-type (Table 1); after 48 h, cultures of the mutant started to bleach. Heterocysts of the mutant had partially detached envelopes, lacked cyanophycin granules at their poles (Fig. 1b), and lacked detectable nitrogenase activity (Table 1). Concordantly, cyanophycin was not detected in strain NE1 that had been deprived of fixed nitrogen for 48 h (Table 1). Thus, interruption of *argL* by the transposon in strain NE1 (see below) and perhaps, as suggested by Gupta & Carr (1981), the resulting absence of cyanophycin granules rendered the structure of the heterocyst abnormal and prevented N₂ assimilation by the heterocysts. An alternative interpretation, that newly fixed NH₃ that is not consumed in the synthesis of arginine may accumulate and uncouple ATP synthesis, and that the heterocyst envelope may become distorted as a consequence of ATP deficiency, appears invalid for the following reason: when wild-type *N. ellipsoforum* and strain NE1 were deprived of fixed nitrogen under an atmosphere of argon, strain NE1 developed heterocysts with the same envelope distortion as observed under N₂-replete conditions, whereas heterocysts of the wild-type strain showed no such distortion.

When strain NE1 was grown in sporulation medium with nitrate, virtually every cell appeared to become an akinete eventually. Like akinetes of the wild-type strain, these cells were larger than vegetative cells, were detached from other cells, and had a thicker envelope than did vegetative cells (Fig. 1e). They appeared at the same time as did akinetes in the wild-type strain. However, they were smaller and less rounded than the wild-type akinetes, had a homogeneous appearance (Fig. 1e) due to the lack of cyanophycin granules, and failed to germinate. A small amount of cyanophycin was detectable analytically (Table 1). Thus, interruption of *argL* by Tn5-1058 (see below), and perhaps the absence of cyanophycin granules, prevented normal functioning of akinetes in *N. ellipsoforum*. Although stored cyanophycin is mobilized during the process of akinete germination, Sutherland *et al.* (1985) concluded that cyanophycin is not the primary nitrogen source for *de*

Table 1. Effect of the nitrogen source on the growth, nitrogenase activity and cyanophycin content of cultures of *N. elliposporum* wild-type and mutant NE1

The culture medium used for growth on N_2 was that of Hu *et al.* (1981), and that for growth on NO_3^- was (HEPES-buffered) standard sporulation medium (Wolk, 1965). The doubling time, nitrogenase activity and cyanophycin content were determined at 48 h, except that the cyanophycin content of cultures grown with NO_3^- and $NO_3^- + 1$ mM Arg (L-arginine) was determined after 20 d culture, when akinetes had differentiated (see Fig. 1). The results shown are the means \pm standard error of the mean of three independent experiments with duplicate samples. ND, Not detected.

<i>Nostoc</i> strain	Nitrogen source	Doubling time (h)	Nitrogenase activity [nmol C_2H_4 (mg DW) $^{-1}$ h $^{-1}$]	Cyanophycin content [μ g L-Arg (mg DW) $^{-1}$]
Wild-type	N_2	41.6 \pm 2.3	159 \pm 13.5	2.3 \pm 0.7
NE1	N_2	65.8 \pm 2.9*	ND*	ND*
Wild type	NO_3^-	23.1 \pm 2.1	ND	4.1 \pm 0.5
NE1	NO_3^-	23.4 \pm 2.1	ND	0.8 \pm 0.2*
Wild type	$NO_3^- + 1$ mM Arg	24.4 \pm 2.1	ND	15.7 \pm 2.0
NE1	$NO_3^- + 1$ mM Arg	25.2 \pm 1.7	ND	4.7 \pm 1.4*

* Differed statistically (Student's *t* test, $P < 0.05$) from the wild-type strain under the same culture conditions.

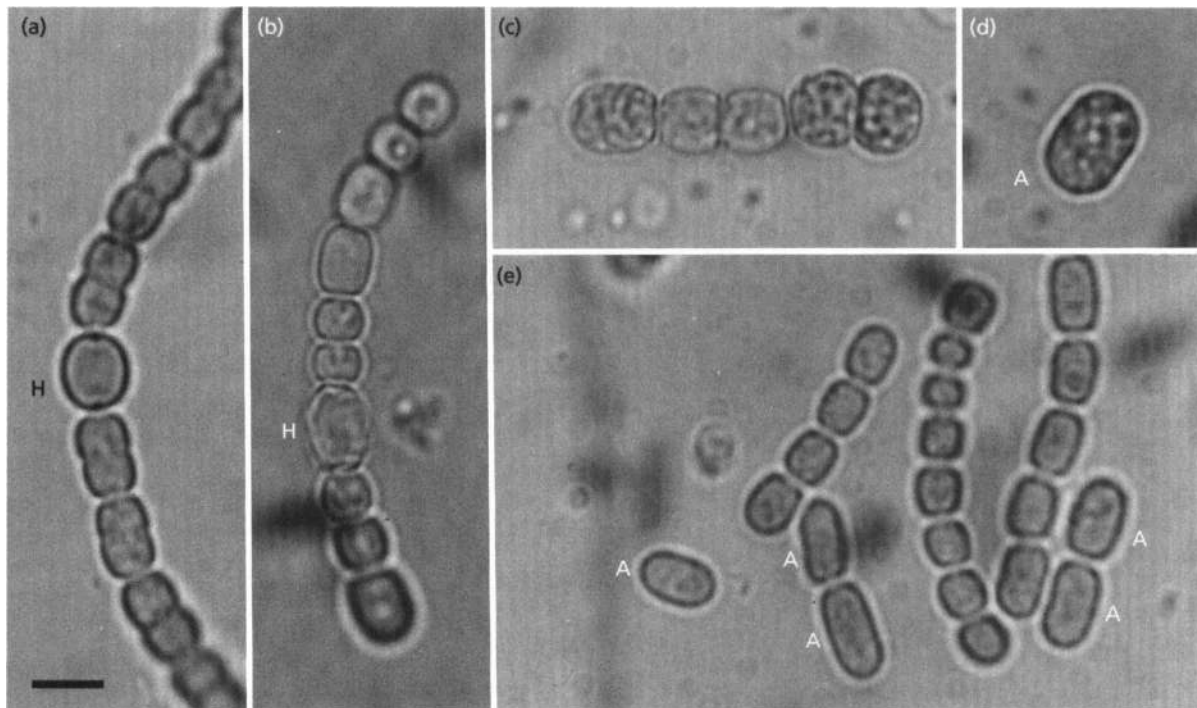


Fig. 1. Heterocysts (H) in wild-type *N. elliposporum* (a) and strain NE1 (b) 48 h after transfer to AA/8 medium lacking both nitrate and arginine. The heterocysts of strain NE1 lack polar cyanophycin granules and have an abnormal envelope. Wild-type *N. elliposporum* (c, d) and NE1 (e) after 20 d growth in standard sporulation medium (which contains nitrate) supplemented with calcium glucuronate. Apparent akinetes (A) in NE1 (e) are smaller and less rounded than akinetes in the wild-type strain (d) and their interior appears homogeneous due to the lack of cyanophycin granules. Whereas vegetative cells of wild-type *N. elliposporum* have visible cyanophycin granules, vegetative cells of NE1 do not (compare c and e). Bar, 5 μ m.

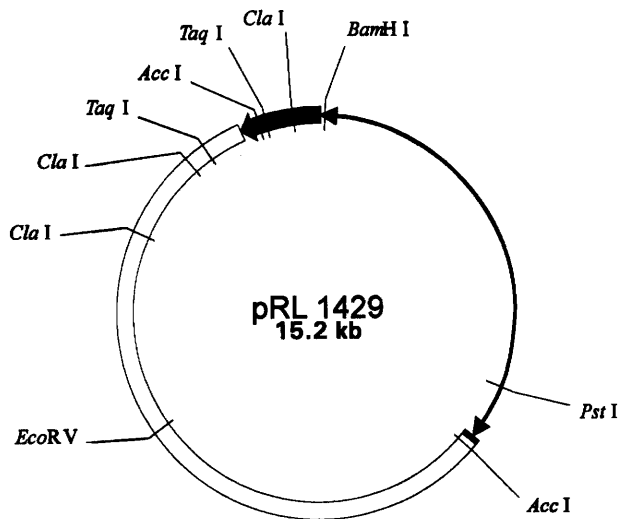


Fig. 2. Physical map of plasmid pRL1429 bearing transposon Tn5-1058 (5.4 kb; thin curved line) together with genomic DNA recovered from the DNA of strain NE1 by restriction with *EcoRV*, ligation, and transfer to *E. coli*. Insertion of the transposon duplicated bp 56–64 in the 1056 bp *argL* gene. Black boxes, *argL*; open box, other *N. ellipsoforum* DNA. Only the positions of restriction sites used for subcloning and sequencing are depicted.

novo synthesis of protein. Therefore, the inability of akinetes of NE1 to germinate may not have been due to a lack of cyanophycin as a nitrogen source for protein synthesis.

In sporulation medium with nitrate, wild-type *N. ellipsoforum* accumulated cyanophycin in the stationary phase of growth (Table 1) with granules visible in vegetative cells and akinetes (Fig. 1c). In contrast,

prolonged incubation of strain NE1 in sporulation medium resulted in death of the culture. Death was prevented if the differentiation of akinetes was avoided by subculturing to fresh nitrate-supplemented liquid medium (normally AA/8 + NO₃⁻; Hu *et al.*, 1981) every 4–5 d (on solid medium, once per week). Thus, unlike *argC* mutants CS335 and CS336 of *Anabaena* sp. strain PCC 7120 (Floriano *et al.*, 1992), NE1 is not a strict L-arginine auxotroph, but rather a conditional auxotroph.

Because the gene mutated in NE1 showed great similarity to a gene involved in the synthesis of L-arginine (see below), growth media were supplemented with this amino acid. When both L-arginine and nitrate were present, strain NE1 formed cyanophycin (Table 1) and small, presumptive akinetes, some of which contained cyanophycin granules. Cultures of strain NE1 in sporulation medium supplemented with L-arginine reinitiated growth when transferred to fresh medium, although normal germination of akinetes was never observed. Addition of L-arginine, as nitrogen source, to a medium lacking combined nitrogen prevented heterocyst differentiation in both the wild-type and strain NE1, and nitrogenase activity was undetectable (data not shown).

Reconstruction of the mutation present in mutant NE1

To determine whether the phenotype of NE1 was the result of insertion of the transposon, rather than the result of a secondary mutation, the transposon insertion was reconstructed (see Black *et al.*, 1993). Transposon Tn5-1058 (5.4 kb) and 9.8 kb of contiguous DNA were recovered from mutant NE1 upon excision with *EcoRV*, circularization, and transfer to *E. coli*, producing plasmid pRL1429 (Fig. 2), from which plasmid pRL1505 was constructed (see Methods). Sequencing outward

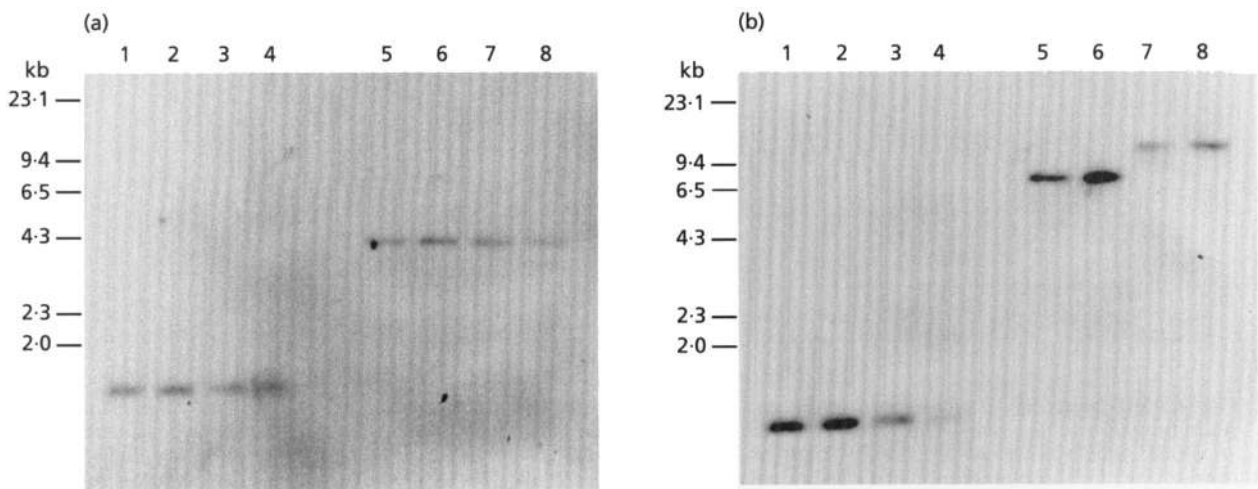


Fig. 3. Southern analysis of DNA from *Anabaena* sp. strain PCC 7120 (lanes 1, 5), wild-type *N. ellipsoforum* (lanes 2, 6), strain NE1 (lanes 3, 7), and strain DR1505 (lanes 4, 8) cut with *ClaI* (lanes 1–4) or *EcoRV* (lanes 5–8), and probed with (a) a 1.6 kb *ClaI* fragment that contains *argC* from *Anabaena* sp. strain PCC 7120 (Floriano *et al.*, 1992) or (b) a 1.3 kb *ClaI* fragment that bears part of the gene mutated in strain NE1 (*argL*; see text). The two probes hybridized to DNA from both sources, but targeted bands of different sizes.

Bs	M KIGIVGATGCGTELVRIILSHHPRHECILLYSSSGEGNVYSEGYPHLTGLADQQLKPIDMNTIK	65
Ne	MCNFGRVFVGVIGVAGSGYGGVQLVRLINDHPEIETLVYGGSSVGKSFASLYPHLAHAVKLSIEEVDPEVIA	71
Ss	MCN . EKARVGIIGASGYGGIQVLRLLLEHPQVELTYLAGHSSAGKPYSDLYPHLTHRVNLTETPIDLEATA	70
An	M . . . NKPKIFIDGAGTGLQIYSRLNERDDIELVSIASKRKDADEKAKLL	49
Bs	HEIDIMFLAAPPVSSSELTTPKLDAGITVIDLSGLDRIKEPAEYERWYKRTAAPKAVIQEAVYGLAELNQL	136
Ne	RRCEVVFLEMPNGLACQIVPTLLEKGCCKVLDLADYRFRNLTYTITWYGVRSORTTADTAIYGLPELYRD	142
Ss	SRCDAVFLGLPNGLACDMPALLAKGCKVLDLADYRFRNLTYTITWYKDRDQATNNQAVYGLPELYRD	141
An	NSVDVAILCLPDDAAREAVSLVNSSQVKILDASTAYRTAQGWVYGFPEMNPQRE	104
Bs	QIQQAQLIANPGCFPTAVLLGLAFLAQKLL . DESFVIVDAKTGVSGAGRKASMGTHFSELNDFNKIYKVN	206
Ne	RISEAQLVGCPSGYPTASILLALSPLLKQGLIVPE . TAIVDAKSGTSGCGREAKTYLLLAADNSLAPYSVV	212
Ss	AIRDAQLIGCGCYPTASILLALSPLLKQGLIVPE . TAIIDAKSGTSGCGREAKVMMLLAAEGSLGAYGVA	211
An	KIANAQFVSNPGCYPTGFLACVRFLIA Q GILPSSFPITINAV SGYSGG CKSLIQKYSDFHEQQKATSDYP	175
An		FGIYGLQFAGH 186
Bs	EHQHTPEIEQALNEWQPLGIPITSAHLFPMTGRIMATMY . TRLTCDLTADDLHDLYSEFYQDSYFVRVRP	276
Ne	RHRHTPEIEQICSDLAGHEVTVQFTPHLVPIVRGILATVYATLRDPLVGGDLTTIYTAFYRNSPWVKVCE	283
Ss	KHRHTPEIEQIASDLAGTELRVQFTPHLIPMVRGILATVYATLRDPLVGGDLTTIYSAFYRASPFVKILP	282
An	KHVKEMHQSGLLFIPAVGDFEQMLVQIPLPLWTLDNPPSGEEIHQAIAQYQGERKVFQVAFPKDPSLLR	257
Bs	KGQYPQTKVEYGSNFCDIARTLDERTRKVTIVSVIDNLMKGAAGQAVONFNLNMGWNEETGLTITPIYP	345
Ne	SGIYPQTKWAAGSNLCYIGVEVDPRTRGRVIGMSVIDNLRGGAGQATQCLINDAGWDETLGLFRMGFYF	352
Ss	HGVYPQTKWANGTNLCYIGIEVDPRTRGRVIVLSAIDNLRGGAGQAVQCLINDAGWEESLGLFQLAFYF	351
An	DGTFELDATAVNGTNIQVVFVFGNDNTKEALLVARIDNLR GK ASRAVONINIMLGLPEELQI	321

Fig. 4. Comparison of the predicted sequences of (Ne) the protein encoded by *argL* from *N. elliposporum* and those of the *N*-acetylglutamate semialdehyde dehydrogenases from (Bs) *B. subtilis* (Smith *et al.*, 1990), (An) *Anabaena* sp. strain PCC 7120 (Floriano *et al.*, 1992), and, by presumption, (Ss) *Synechocystis* sp. strain PCC 6803 (Kaneko *et al.*, 1996). The bold-face characters indicate amino acid residues that are identical in the predicted protein from *N. elliposporum*. The underlined regions are presumptively involved in the binding of nucleotides: the upper two are similar to the 'P-loop' in ATP- and GTP-binding proteins (Floriano *et al.*, 1992; Saraste *et al.*, 1990); the lower three are collectively similar to the $\beta\alpha\beta$ fold involved in binding of NAD, NADP and FAD by some oxidoreductases (Floriano *et al.*, 1992). The asterisk indicates a cysteine residue conserved in *Anabaena* sp. strain PCC 7120, *B. subtilis* and *Synechocystis* sp. strain PCC 6803, but replaced by a serine in *N. elliposporum*.

from the transposon in pRL1429 showed no vestige of pRL1058 outside of the transposon, indicating that strain NE1 was derived from a true transposition of Tn5-1058 (data not shown). Colonies derived from presumptive double recombination of pRL1505 with the wild-type strain were shown by Southern hybridization to have *EcoRV* fragments that differed from those of the wild-type strain but matched that of the original mutant strain NE1 (Fig. 3b). The phenotype of these colonies also matched that of strain NE1. One such strain was designated DR1505.

Analysis of the gene mutated in strain NE1

The transposon in strain NE1 was found to interrupt an open reading frame of 1056 bp encoding a protein of 352 amino acids (Fig. 4) that shows 40% identity (58% similarity) to *N*-acetyl- γ -glutamyl-phosphate reductase (*N*-acetylglutamate semialdehyde dehydrogenase), the product of the arginine biosynthetic gene, *argC*, from *Bacillus subtilis* (Smith *et al.*, 1990); 72% identity (84% similarity) to the hypothetical product of what is presumed to be *argC* of *Synechocystis* sp. strain PCC 6803 (Kaneko *et al.*, 1996); but only 18% identity (26% similarity) to the predicted product of *argC* from *Anabaena* sp. strain PCC 7120 (Floriano *et al.*, 1992). The amino acid sequences deduced from *argC* of *N. elliposporum* (Ne), *Anabaena* sp. strain PCC 7120 (An) and *B. subtilis* (Bs), and of presumptive *argC* of *Synechocystis* sp. strain PCC 6803 (Ss) are compared in Fig. 4. Southern analysis of DNA from *Anabaena* sp. PCC 7120, *N. elliposporum* wild-type, strain NE1 and strain DR1505 using the *argC* genes from *Anabaena* sp. PCC 7120 (Floriano *et al.*, 1992) and *N. elliposporum* (this paper) as probes showed different patterns of hybridization (Fig. 3): one copy of each gene is

present in each of the cyanobacteria. We denote the newly identified gene from *N. elliposporum*, *argL*.

Highly conserved regions (underlined in Fig. 4) of these proteins are similar to domains involved in the binding of nucleotides (Floriano *et al.*, 1992; Saraste *et al.*, 1990). Two domains that resemble a 'P-loop' (ATP-/GTP-binding site motif) are present in ArgL of *N. elliposporum* (amino acids 39–46 and 184–192). A presumptive 'P-loop' in the C-terminal part of the protein is common to all four predicted proteins depicted in Fig. 4, but a second presumptive 'P-loop' in the N-terminal part of the protein is present only in ArgL and in the protein from *Synechocystis* sp. strain PCC 6803. A $\beta\alpha\beta$ fold that is involved in the binding of the adenine nucleotide moiety of NAD, NADP and FAD by some oxidoreductases is present in all four proteins. The cysteine that has been proposed to be involved in the reaction mechanism of *N*-acetylglutamate semialdehyde dehydrogenase (Fig. 4, asterisk; Parsot *et al.*, 1988) is substituted by serine S155 in *N. elliposporum* but may be functionally replaced by the nearby cysteine C152.

As in the case of numerous genes from heterocyst-forming cyanobacteria, the open reading frame that is interrupted by the transposon in NE1 is positioned close to a series of repeated sequences, here 3' from the gene, that may possibly serve as binding sites for regulatory proteins (Holland & Wolk, 1990). A 7 nt repeat (located 65 bp after the termination triplet), CCCAGT, occurring in five perfect and two imperfect sets, differs from that found 3' from *argC* in *Anabaena* sp. strain PCC 7120, CTAATGA (Floriano *et al.*, 1992), but closely resembles the sequence CCCAAT found in *Anabaena* sp. strain PCC 7120 downstream from *hepA* (Holland & Wolk, 1990; gene renamed by Ernst *et al.*, 1992). Also, 10 bp after the termination triplet of *argL*

and prior to the CCCAGT repeats are three consecutive repetitions of the sequence GGGTATT.

Like insertions within *hetR* and *hepA* (Leganés *et al.*, 1994; Leganés, 1994), a transposon insertion in *argL* blocks normal differentiation and function of heterocysts and akinetes. Our results are consistent with the idea that *N. ellipsoforum* has two different genes, *argC* and *argL*, whose products are involved in the biosynthesis of arginine. We suggest that the product of *argC* is involved primarily in the synthesis of arginine for protein biosynthesis whereas the product of *argL* may be involved specifically in formation of cyanophycin.

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