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

F. Leganés, F. Fernández-Piñas and C. P. Wolk

Author for correspondence: F. Leganés. Tel: +34 91 397 8176. Fax: +34 91 397 8344. e-mail: francisco.leganes@uam.es

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. ellipsosporum, 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. ellipsosporum 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. ellipsosporum (Leganes 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 Nrdl and Scal, 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. ellipsosporum 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 pUC19 (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. ellipsosporum 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. ellipsosporum 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. ellipsosporum. 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. ellipsosporum wild-type and mutant NE1

The culture medium used for growth on N₂ was that of Hu et al. (1981), and that for growth on NO₃ 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 NO₃ and NO₃ + 1 mM Arg (l-arginine) was determined after 20 d culture, when akinetes had differentiated (see Fig. 1). The results shown are the means ± standard error of the mean of three independent experiments with duplicate samples. ND, Not detected.

<table>
<thead>
<tr>
<th>Nostoc strain</th>
<th>Nitrogen source</th>
<th>Doubling time (h)</th>
<th>Nitrogenase activity [nmol C₃H₄ (mg DW)⁻¹ h⁻¹]</th>
<th>Cyanophycin content [μg l-Arg (mg DW)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type N₂</td>
<td>41.6 ± 2.3</td>
<td>159 ± 13.5</td>
<td>2.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>NE1 N₂</td>
<td>65.8 ± 2.9*</td>
<td>ND*</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Wild type NO₃</td>
<td>23.1 ± 2.1</td>
<td>ND</td>
<td>4.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>NE1 NO₃</td>
<td>23.4 ± 2.1</td>
<td>ND</td>
<td>0.8 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>Wild type NO₃</td>
<td>24.4 ± 2.1</td>
<td>ND</td>
<td>15.7 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>NE1 NO₃ + 1 mM Arg</td>
<td>25.2 ± 1.7</td>
<td>ND</td>
<td>4.7 ± 1.4*</td>
<td></td>
</tr>
</tbody>
</table>

*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. ellipsosporum (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. ellipsosporum (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. ellipsosporum have visible cyanophycin granules, vegetative cells of NE1 do not (compare c and e). Bar, 5 μm.
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. ellipso sporum* 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, ligation, and transfer to *E. coli*, producing plasmid pRL1429 (Fig. 2), from which plasmid pRL1505 was constructed (see Methods). Sequencing outward
The transposon in strain NE1 was found to interrupt an open reading frame of 1036 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., 1992); 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. ellipso sporum (Ne), Anabaena sp. strain PCC 7120 (An) and B. subtilis (Bs), and of presumptive argC of Synechocystis sp. strain PCC 6803 (Ss) are similar to four predicted proteins depicted in Fig. 3. The underlined regions are similar to the βββ fold involved in the binding of NAD and NADP and FAD by some oxidoreductases (Flohran 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. ellipso sporum.

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), CCCCCAGT, occurring in five perfect and two imperfect sets, differs from that found 3′ from argC in Anabaena sp. strain PCC 7120, CTATAGA (Flohran et al., 1992), but closely resembles the sequence CCCCCAT found in Anabaena sp. strain PCC 7120 downstream from hepaA (Holland & Wolk, 1990; gene renamed by Ernst et al., 1992).
and prior to the CCCCGT repeats are three consecutive repetitions of the sequence GGTATT.

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. ellipsosporum 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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Mutant of Nostoc affected in synthesis of arginine


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