Induction and Differentiation of Heterocysts in the Filamentous Cyanobacterium *Cylindrospermum licheniforme*

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The fragmentation of *Cylindrospermum licheniforme* filaments resulted in the induction of a synchronous round of heterocyst differentiation. Young heterocysts (proheterocysts) appeared 12 to 15 h after fragmentation, and 12 to 15% of the cells became heterocysts within 24 h. NH₄Cl or NaNO₃ prevented the formation of new heterocysts, while differentiation was stimulated twofold by the absence of atmospheric N₂. The sequence of appearance of heterocyst-specific characteristics was observed following filament fragmentation. Nitrogenase activity increased rapidly between 13 and 26 h, paralleling the increase in heterocyst frequency. Glycolipids unique to the heterocyst envelope were also synthesized between 13 and 26 h, and activities of 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase increased after 10 h and 90 h respectively. Aged medium induced sporulation in intact filaments within 1 d, but sporulation could occur no earlier than 2 d after filament fragmentation, indicating that the induction of sporulation requires the presence of mature (but not necessarily functional) heterocysts.

**INTRODUCTION**

Heterocysts and spores (akinetes) differentiate from vegetative cells in several groups of the Gram-negative filamentous cyanobacteria (Stanier & Cohen-Bazire, 1977). These two types of functionally specialized cells have thick envelopes consisting of the same unique polysaccharide (Cardemil & Wolk, 1976). Heterocysts are the site of N₂ fixation under aerobic conditions (Haselkorn, 1978), while spores are perennating bodies which can resume growth under favourable conditions (Rother & Fay, 1977; Yamamoto, 1975, 1976). Although heterocyst differentiation has been investigated in many species, there have been few studies of the relationships between these alternative pathways of differentiation (Nichols & Carr, 1978; Sutherland et al., 1979). There is only one cyanobacterium, *Cylindrospermum licheniforme*, in which it is currently possible to induce a round of sporulation by the addition of an exogenous compound found in the medium of aged [sporulating] cultures (Hirosawa & Wolk, 1979a, b). Heterocyst formation has not been previously studied in *Cylindrospermum*, and in this paper we report the development of standard conditions for the reproducible induction of heterocyst differentiation in this species.

Heterocysts occupy only terminal positions in filaments of *C. licheniforme*. Since the link between a heterocyst and a vegetative cell is more fragile than the link between two vegetative cells, filament breakage produces short vegetative filaments lacking heterocysts. One or both of the newly terminal vegetative cells then differentiates into a heterocyst (Wolk, 1965). We have examined the changes in heterocyst-specific characteristics during a round of heterocyst differentiation induced by fragmentation. These characteristics include: the nitrogenase system; glycolipids unique to the heterocyst envelope; and enzymes of the pentose phosphate

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pathway. In addition, we have used the inducer of sporulation present in aged (conditioned) medium to study the relationship between heterocyst and spore formation.

METHODS

Organism and culture. Cylindrospermum licheniforme (Kütz.) (UTEX B 1828) was grown axenically at 32 °C in Allen & Arnon's (1955) N-free medium diluted 16-fold (AA/16). Cultures were inoculated at 1% (v/v) from 7-d-old cultures and incubated on a gyratory shaker (150 rev. min⁻¹) under continuous illumination by cool white fluorescent lights (600 ft-candle; about 6500 lx). Cell number increased exponentially over a period of 7 d with a doubling time of about 24 h. Cultures were routinely tested for contamination by plating a 0-1 ml sample on 1% (w/v) agar plates containing 5 g NaCl and 10 g Bactotryptone l⁻¹. The plates were incubated for 1 week at 32 °C.

Heterocyst induction. Cultures were sonicated at room temperature four times for 0.15 s ml⁻¹ with a microtip of a Branson sonifier at 30 W. The cultures were then incubated as described above. Morphological changes were observed with a light microscope at 256× magnification. A proheterocyst was defined as an enlarged terminal cell, and a mature heterocyst as an enlarged terminal cell with a pore plug at its connection to an adjacent vegetative cell.

When required, stock solutions of 1.0 mM-D,L-methionine sulfoximine and 250 μM-7-azatryptophan were sterilized by Millipore filtration, and solutions of 50 mM-NH₄Cl and 590 mM-NaNO₃ in 10-fold concentrated AA/16 were autoclaved.

Nitrogenase activity. Acetylene reduction was used as an assay for nitrogenase activity (Stewart et al., 1968). Cell suspensions containing 0.5 × 10⁸ to 1.0 × 10⁸ cells ml⁻¹ in 10 ml medium were placed in 50 ml Erlenmeyer flasks and sealed with serum stoppers. Acetylene gas (5-9 ml) was added and 0.3 ml gas samples were withdrawn at various times and analysed on a Tracor MT-220 (Tracor, Austin, TX 10423, U.S.A.) gas chromatograph equipped with a flame detector. The components in the samples were separated at 50 °C on a column of Poropak R, 80-100 mesh (Applied Science Laboratories, Ann Arbor, MI 48107, U.S.A.).

Analysis of heterocyst glycolipids. Samples containing 10³ to 10⁷ cells were extracted twice with 5.0 ml methanol/chloroform (1:2, v/v). The combined extract was blown dry with N₂ and the residue was dissolved in 0.2 ml methanol/chloroform (1:2, v/v). Samples were spotted on a 10 × 20 cm thin-layer plate pre-coated with 0.26 mm of F-254 silica gel (EM Laboratories, Elmsford, NY 10523, U.S.A.), and chromatographed in a solvent of chloroform/methanol/acetic acid/water (170:30:20:7.4, by vol.; Winkenbach et al., 1972). Glycolipids were identified by charring. The plate was sprayed with a mixture containing 1.5 ml 15% (w/v) ethanolic α-naphthol, 6.5 ml concentrated sulphuric acid, 4.05 ml 96% ethanol and 4 ml water, and then heated at 100 °C for 3 to 6 min. (Krebs et al., 1969).

For measurement of glycolipid synthesis, [1⁴C]acetate [2.5 μCi; 50 mCi mmol⁻¹ (1.85 GBq mmol⁻¹); ICN Radiochemicals, Irvine, CA 92714, U.S.A.] was added to cell suspensions (5 × 10⁸ cells ml⁻¹ in 15 ml medium) in 50 ml Erlenmeyer flasks. After incubation for 3 h, cells were extracted with methanol/chloroform (2:1, v/v) and portions of extracts containing 20000 c.p.m. were separated by thin-layer chromatography (t.l.c.). The thin-layer plate was exposed to Kodak RP-5 X-ray film for 2 weeks at −20 °C and then developed at room temperature with Kodak Liquid X-ray developer (4 min), water as stop (1 min), and Kodak Rapid Fix (8 min). The exposed film was then scanned at 540 nm using a Gilford Spectrophotometer. The percentage of total lipid radioactivity in heterocyst-specific glycolipids was determined by integration of the appropriate peaks in the spectrophotometer tracings.

Measurement of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44). Samples containing 2 × 10⁷ cells were centrifuged at 1700 g, washed once with 0.1 M-potassium phosphate buffer (pH 7.0), and resuspended in 2 ml buffer. Cells were sonicated at 4 °C eight times for 15 s each (with 15 s cooling periods between treatments) at 30 W with a Branson sonifier microtip. Using this procedure 90% of the heterocysts and 99% of the vegetative cells were broken in a typical experiment. The sonicate was centrifuged (10000 g, 15min), and the supernatant used in the enzyme assays. The 3 ml assay mixtures contained 0.1 M-potassium phosphate buffer, 0.75 μmol NADP, cell-free extract (5 to 10 μg protein), and 5 μmol glucose-6-phosphate or 6-phosphogluconate (Pearce & Carr, 1969). The enzyme was assayed at room temperature, and the rate of NADPH production, measured as the change in absorbance at 340 nm, was linear with both time and the amount of cell-free extract. Since the product of glucose-6-phosphate dehydrogenase (6-phosphogluconate) is a substrate for 6-phosphogluconate dehydrogenase, the presence of 6-phosphogluconate dehydrogenase in the cell-free extract could produce an illusory increase in the rate of NADPH production by glucose-6-phosphate dehydrogenase. However, the Kₘ of 6-phosphogluconate dehydrogenase for 6-phosphogluconate was 209 μM in the assay mixture (Van de Water, 1979). Thus, the amount of 6-phosphogluconate produced by glucose-6-phosphate dehydrogenase under the assay conditions would only allow 6-phosphogluconate dehydrogenase to produce NADPH at an undetectable rate of 2·8×10⁻⁶ A₃₄₀ units min⁻¹.
Protein was assayed by the Lowry procedure using bovine serum albumin as a standard. Enzyme localization was determined using the indirect-controlled sonication method of Wolk & Wojciuch (1971).

Measurement of glutamine synthetase (EC 6.3.1.2). The assay mixture (1 ml) contained: 40 mm-imidazole buffer, pH 7.0; 30 mm-glutamine; 3 mm-MnCl2; 0.4 mm ADP sodium salt, pH 7.0; 20 mm-potassium arsenate; 60 mm hydroxylamine, HCl, pH 7.0; and cell-free extract (20 to 50 µg protein) (Skapiro & Stadtman, 1970). Reaction mixtures were incubated at 37 °C for up to 30 min. Reactions were terminated at 2 to 5 min intervals by the addition of 2 ml stop mixture containing 3.5% (w/v) FeCl3, 2.0% (w/v) trichloroacetic acid and 0.25% (v/v) HCl. The γ-glutamylhydroxamate produced by the enzyme was monitored by measuring the absorbance at 540 nm. The rate of increase in A540 was linear with both time and the amount of cell-free extract.

Sporulation. Because few spores formed after 2 weeks growth in AA/16, cultures were grown in a modification of Wolk's standard sporulation medium (SSM) (Fisher & Wolk, 1976). SSM lacking NO3 (SSM − NO3) was also used in some experiments so that heterocyst differentiation would not be inhibited. For the production of conditioned SSM or conditioned SSM − NO3, 5-litre diphtheria bottles containing 3 l SSM or SSM − NO3 were inoculated with 100 ml of a 7 d culture grown in AA/16. The cultures were maintained at 32 °C, illuminated with fluorescent light (600 ft-candle) and aerated at a rate of 4 l min−1. Sporulation began after 1 week, but the medium with the maximum stimulatory potential was harvested after 2 to 3 weeks, a period when sporulation was extensive, cell pigments were bleached, and cell number was declining. The cells were removed from the medium by low-speed centrifugation. The supernatant was concentrated 10-fold in a flash evaporator at 40 °C and stored at −20 °C. Conditioned SSM or SSM − NO3 was autoclaved before use. Sporulation was induced when the concentrated medium was added at a dilution of 10% (v/v) to cells growing in AA/16.

RESULTS

Heterocyst differentiation

Following controlled filament fragmentation, heterocysts differentiated rapidly and synchronously (Fig. 1). Terminal cells started to enlarge between 9 and 12 h after fragmentation. These proheterocysts increased in size and number up to 15 h, and then declined in frequency as they were transformed into mature heterocysts. In 89% of all experiments, mature heterocysts appeared after 15 ± 1 h. The heterocyst to vegetative cell ratio levelled off at 24 h. However, the total heterocyst number in the culture continued to increase so that 50% and 70% of the terminal cells were heterocysts at 25 and 34 h, respectively. The greatest number of heterocysts per 100 cells differentiated after filaments were fragmented to an average length of 3 to 6 cells (results not shown). Vegetative growth in cell number and filament length was inhibited 90% by fragmentation, but resumed after 24 h. After 24 h, the heterocyst frequency gradually declined to the level of 2 to 3% usually found in unfragmented growing cultures.

Addition of NO3− or NH4+ (0.5 to 5.0 mM) to growing or fragmented cultures resulted in a suppression of heterocyst differentiation. However, heterocyst formation was not induced by compounds which interfere with nitrogen assimilation. Methioninesulphoximine (0.1 mM), an inhibitor of glutamine synthetase in Cylindrospermum (Meeks et al., 1978), stopped growth, prevented new heterocysts from forming, and caused mature heterocysts to detach. 7-Azatryptophan at 5 or 10 µM had no effect on heterocyst frequency in either intact or fragmented filaments. At 25 µM, it allowed heterocyst frequency to increase slightly in intact filaments; an occasional intercalary heterocyst was seen in some of the filaments, but no groups of two or more adjacent heterocysts were observed. When fragmented filaments were incubated in an atmosphere of Ar/O2/CO2 (80:20:0.04, by vol.), heterocyst frequency increased 1.5- to 2-fold relative to filaments in control cultures incubated in air. Under these conditions of nitrogen starvation vegetative cell division did not resume after 24 h.

Markers of heterocyst differentiation

Sonication reduced attached heterocyst frequency by 70% and total nitrogenase activity by 80%. Between 13 and 26 h after filaments were fragmented, nitrogenase activity per 10⁶ cells increased sixfold, while heterocyst frequency increased fourfold (Fig. 1). After 24 h, nitrogenase activity continued to increase slowly, while heterocyst frequency declined as vegetative growth resumed.
Fig. 1. Frequency of differentiated cells (---) and nitrogenase activity (-----) as a function of time after the fragmentation of *C. licheniforme* filaments. Heterocyst (●) and proheterocyst (○) frequencies are given as a percentage of the total cells in the culture. The results are a composite of two separate experiments.

Fig. 2. Percentage of total labelled chloroform/methanol-soluble material in heterocyst-specific glycolipids as a function of time after fragmentation of *C. licheniforme* filaments.

The three unique glycolipids present in *Anabaena* heterocysts (Winkenbach et al., 1972) are also present in *Cylindrospermum* heterocyst extracts and can be separated by t.l.c. The incorporation of radioactivity from [14C]acetate was used as an assay for the timing of synthesis of this class of lipids following fragmentation-induced heterocyst differentiation. [14C]Acetate was used as the labelling compound because 75% of the 14C incorporated was soluble in methanol/chloroform (2:1, v/v). The heterocyst glycolipids (Fig. 2) were synthesized between 13 and 24 h following filament fragmentation. The heterocyst glycolipid which migrated the farthest was never made in amounts large enough to be distinguishable from the background incorporation at the same R<sub>r</sub> value. However, the other two glycolipids were synthesized in a constant ratio throughout differentiation.

Contrary to expectation (Stanier & Cohen-Bazire, 1977), the specific activity of glucose-6-phosphate dehydrogenase was unchanged if Mg<sup>2+</sup> or ATP was added to the reaction mixture, or if the cell-free extracts were diluted or preincubated with glucose-
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Fig. 3. Percentage activity of 6-phosphogluconate dehydrogenase (■) and glucose-6-phosphate dehydrogenase (□) remaining bound to cells during the controlled sonication of *C. licheniforme* filaments. The percentages of heterocysts (○) and vegetative cells (●) remaining intact under the same conditions are also presented. Enzyme activities are plotted as percentages of total activity which could possibly be released at infinite time, but still remain in a cell pellet at time *t*. The extent of sonication treatment is proportional to the sonifier power output, and inversely proportional to the solution volume. Therefore, the abscissa represents the sums of successive times of sonication per ml of cell suspension which remained after each consecutive sample was removed.

Fig. 4. Specific activities of glucose-6-phosphate dehydrogenase (○) and 6-phosphogluconate dehydrogenase (■) as a function of time after *C. licheniforme* filaments were fragmented. Activities are given as nmol NADPH min⁻¹ (mg protein)⁻¹ and as nmol NADPH min⁻¹ (10⁶ cells)⁻¹, respectively. The arrows *P* and *H* in the figure indicate the times of maximum proheterocyst (*P*) and heterocyst (*H*) differentiation.

6-phosphate. Although a constant concentration of enzyme in the assay was not critical, approximately the same enzyme activities were used in all assay mixtures.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were both localized in heterocysts. Controlled sonication destroyed vegetative cells faster than heterocysts, but both enzymes were solubilized more slowly than vegetative cells were destroyed (Fig. 3). If the slow-release portion of the plot of enzyme activity is extrapolated back to zero time, the ordinate value equals the percentage of total activity which is localized in heterocysts (Wolk & Wojciuch, 1971). It was calculated that 92% of glucose-6-phosphate
Fig. 5. Timing of sporulation during a round of heterocyst differentiation induced by filament fragmentation of *C. licheniforme*. Filaments were fragmented in AA/16, and a 0.1 volume of 10-fold concentrated conditioned SSM lacking NO$_3$ was added either immediately (○) or after 24 h (●). Control cultures contained no additions (□). For comparison, 10-fold concentrated conditioned SSM was added to an unfragmented culture (■). At each time period indicated, the samples were scored for the presence of spores. In all cases spores formed adjacent to heterocysts. The extent of sporulation is given as spores present in the culture per 100 heterocysts.

Spores appeared within 1 d after cells were inoculated into conditioned SSM or after 0.1 volume of 10-fold concentrated, conditioned SSM — NO$_3$ was added to exponential-phase cells growing in AA/16. Sporulation began 2 d after filaments were fragmented, whether conditioned medium was added at 0 or 24 h (Fig. 5). Thus heterocysts must be present before spores will form. The infrequent spores which formed in the absence of conditioned SSM — NO$_3$ occurred adjacent to those few attached heterocysts that had survived fragmentation.

Sporulation did not require heterocysts that were actively fixing N$_2$. When SSM — NO$_3$ was added immediately after filaments were fragmented and the cultures were then bubbled with Ar/O$_2$/CO$_2$ (80:20:0.04, by vol.), the number of spores per hundred cells was similar to that in control cultures incubated in air. Fragmented filaments grown under Ar/O$_2$/CO$_2$ formed few spores in the absence of conditioned medium.
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DISCUSSION

Heterocyst induction systems in Anabaena cylindrica, Anabaena 7120 and Anabaena CA have involved the transfer of intact filaments from N-containing to N-free medium (Haselkorn, 1978). Although mature heterocysts are largely absent from filaments of A. cylindrica grown in the presence of NH₄⁺, Wilcox et al. (1973a) observed proheterocysts located in the usual heterocyst pattern. Heterocysts induced by transfer may thus differentiate from vegetative cells which have already passed through some of the earliest stages of development. In contrast, most of the heterocysts newly formed following fragmentation of C. licheniforme would not have differentiated in intact filaments because two to four times more heterocysts per ml were formed in the first 24 h than in an unsonicated control culture. Filament fragmentation may therefore be useful for studying the early stages of heterocyst differentiation.

As in Anabaena sp. (Haselkorn, 1978), the initial signal for the induction of heterocyst differentiation in C. licheniforme is probably transient N starvation. Growth in N-containing medium represses nitrogenase and, as in other species, sonication drastically reduces nitrogenase activity in C. licheniforme (Wolk, 1970; Weare & Beneman, 1973). Cell division ceased for 24 h after filaments were fragmented and, if atmospheric N₂ were present, resumed at the same time as nitrogenase activity rose.

Cylindrospermum heterocysts differentiated at about the same rate as those in Anabaena. Proheterocysts appeared after 6 h in A. cylindrica and after 9 to 12 h in C. licheniforme: mature heterocysts with polar plugs appeared after 16 h in both species (Wilcox et al., 1973a, b). Heterocysts differentiated more slowly in older cultures than in younger cultures of both species, and only incubation of fragmented cultures in the absence of N₂ stimulated heterocyst differentiation. Unexpectedly, neither 7-azatryptophan nor D,L-methioninesulphoximine induced heterocysts in fragmented filaments. A five-fold higher concentration of 7-azatryptophan was needed to stimulate even slightly heterocyst frequency in Cylindrospermum than was required to stimulate differentiation substantially in other species (Mitchison & Wilcox, 1973).

As in filaments of Anabaena 7120 (Fleming & Haselkorn, 1974), nitrogenase activity and heterocyst frequency increased at the same time. In several Anabaena species, inactive nitrogenase polypeptides are synthesized before the system which protects nitrogenase from O₂ is complete (Fleming & Haselkorn, 1974; Rippka & Stanier, 1976, 1978). Lambein & Wolk (1973) suggested that the heterocyst-specific glycolipids limit the influx of O₂ into mature heterocysts. The data reported here support this contention because heterocyst glycolipids in C. licheniforme were synthesized at the same time as nitrogenase activity increased.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase both appeared to be localized in the heterocyst. In other species these enzymes have been measured in gently isolated heterocysts (with intact pore regions) and intact filaments (Winkenbach & Wolk, 1973). The gentle methods do not lyse vegetative cells of C. licheniforme and heterocysts must be isolated from filaments by extensive sonication (results not shown). This ruptures the pore channels (Fay & Lang, 1971) and may allow heterocyst contents to leak out. Indeed, gentle sonication released both enzymes slightly faster than heterocysts were destroyed. Thus, the indirect method of Wolk & Wojciuch (1971) was used to estimate the proportion of total enzyme activity in heterocysts. The enzymes were concentrated 400- and 100-fold, respectively, in heterocysts on a per cell basis. These numbers must be regarded as approximate, since intercept values varied by as much as 20% between experiments. It is unlikely that changes in effector levels were responsible for changes in activities of either enzyme during differentiation, since enzyme activities were additive when assays contained mixtures of cell-free extracts made from filaments at different times during heterocyst differentiation.
Subterminal cells of *C. licheniforme* sporulated rapidly and synchronously in the presence of conditioned SSM (Fisher & Wolk, 1976). Although mature heterocysts must be present before spores will form, this requirement is not related to heterocyst \( \text{N}_2 \)-fixing functions, since (1) spores formed at a normal rate in the absence of exogenous \( \text{N}_2 \), and (2) spores never formed until heterocysts were at least 2 d old, long after the new heterocysts began to fix \( \text{N}_2 \).

The results presented here are consistent with the concept that there are at least two groups of events in heterocyst maturation (Fig. 6): one set which occurs between the time of induction and the appearance of active nitrogenase activity (0 to 30 h), and a second set which includes the ability to induce sporulation and takes place after the newly differentiated heterocyst is capable of fixing \( \text{N}_2 \). The reappearance of phycobiliproteins in heterocysts of *Anabaena* sp. L-31 described by Thomas (1972) may also be in the latter class of events. Since 75% of the heterocysts in an exponentially growing culture are between 0 and 2 generations old (Simon, 1980), any event occurring later in the heterocyst life cycle would not be seen until older heterocysts had accumulated in non-growing (stationary phase) cultures.

In summary, these studies have established conditions for reproducible heterocyst differentiation in a species in which both spore and heterocyst formation can be studied. The timing of several important events of heterocyst differentiation was determined, and even though the heterocyst pattern is different from that previously studied (terminal vs. intercalary), the process was shown to be similar to that observed in other cyanobacteria. This species will be a useful model system for the study of prokaryotic cell differentiation.

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**REFERENCES**

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