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Characterization of the *Calothrix* isolates from rice fields

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Abstract. Seven *Calothrix* isolates which can be differentiated by the relative electrophoretic mobility of their phosphoglucose isomerase (EC:5,3,1,9) and their phosphogluconate dehydrogenase (EC:1,1,1,44) during gel-electrophoresis were studied. Based on the morphology of the basal filament region during development, *Calothrix* HC101, HC205, HC301, and HC401, were grouped together as "cylindrical types", whereas *Calothrix* HC601, HC605, and HC701 were considered to be "coryneform types". The morphological and physiological features of the seven isolates were compared. The α-ketoglutarate dehydrogenase activities of the seven isolates were either non-detectable or very weak in despite of they could grow heterotrophically. The nitrogenase activity of all the *Calothrix* isolates was completely repressed by 0.1% sodium nitrate. The nitrate-repression of the nitrogen fixation of the coryneform types was partially relieved by 0.5% glucose. A similar glucose derepression effect was not observed with the cylindrical types. A "close-contact" orientation of the hormogonia of *Calothrix* HC605 was also observed.

Key words: *Calothrix*; Close-contact; Heterotrophic growth; α-Ketoglutarate dehydrogenase; Morphological feature; Nitrogen-fixation; Physiological property; Zymograms.

Introduction

Calothrix is widely distributed and ecologically abundant. During a survey conducted in the Philippines, India, Malaysia and Portugal, *Calothrix* was always found to be present in rice fields with the populations slightly smaller than those of *Nostoc* and *Anabaena* (Roger *et al.*, 1987). During a survey of the nitrogen-fixing cyanobacteria in Taiwan rice fields, *Calothrix* was also found to be one of the major groups. Reports on the structure, morphology and nitrogen-fixing activity of *Calothrix* have recently been reviewed by Whitton (1987). From it, the relative paucity of information on the physiological and biochemical properties of *Calothrix* compared to *Nostoc* or *Anabaena* is obvious. In the present study, some strains of *Calothrix* were isolated from local rice paddy fields and we now report on their morphology and some of their physiological and biochemical properties of axenic cultures.

Materials and Methods

Isolation and Purification

BG-11 medium (Stanier *et al.*, 1971) devoid of sodium nitrate (BG-llo medium) was used for the isolation and purification of *Calothrix* according to the procedures described previously (Huang and Chow, 1983). The purity of cultures was checked by culturing in nutrient broth and by microscopic examination. The axenic cultures were maintained on BG-llo slants containing 1.2% agar and 0.1% active carbon. The cultures were subcultured at intervals of about 3 months.

Cultivation of Cyanobacteria

Axenic *Calothrix* cultures were cultivated without shaking in 125 ml Erlenmeyer flasks containing 30 ml BG-llo medium, supplemented with 0.01M N-(2-hydroxyethyl) piperazine-N'-3-propanesulfonic acid (EPPS) buffer, pH 8.0. They were grown in continuous white

fluorescent light (Toshiba FL 20D/18, Taiwan Fluorescent Lamp Co.). Unless stated otherwise, they were incubated at 28°C under about 35 μmol photon • m^{-2} • s^{-1}. The growth rate of the *Calothrix* isolates were estimated according to the procedure described previously (Huang and Chow, 1988).

For the dark heterotrophic growth, the *Calothrix* culture growing in EPPS-buffered BG-llo were supplemented with 1% of either D-arabinose, D-fructose, D-glucose, I-inositol, lactose, mannitol, L-rhamnose, D-ribose, sucrose, D-xylose, sodium acetate, sodium citrate or sodium succinate respectively. The sugars or other organic energy sources were prepared separately as 10% stock solutions and sterilized by filtration before they were added to the 0.01 M EPPS-buffered BG-llo. The *Calothrix* isolates were transferred from agar slants to the liquid medium by loop. The cultures were cultivated in darkness without shaking at 28°C. The ability of the *Calothrix* isolates to grow in the dark was examined at 2 week intervals for a period of 2 months.

Observations on Trichrome Development

A simplified microculture technique was used to study the morphological changes of the *Calothrix* isolates that occurred with time. Three ml of the melted EPPS-buffered BG-llo agar was smeared on a sterilized slide. A small amount of cyanobacterial material was spread over the solidified agar surface. The slide was kept inside a petri dish which also contained wet cotton wool to ensure a high humidity. The petri dish was incubated at the same temperature and at the same light intensity as stated at the "cultivation" section. The growth and the morphological development of the *Calothrix* isolates were microscopically examined at regular intervals.

*Chlorophyll **a** Content*

Chlorophyll *a* was extracted quantitatively with 100% methanol (Merck) at 60°C for 30 min and determined at 663 nm using 78.74 l/g • cm as the specific absorption coefficient (Codd *et al.*, 1980). Absorption spectra were obtained on an Uvikon 810 spectrophotometer.

Assay of Nitrogenase Activity

Nitrogenase activity was assayed by the acetylene reduction method (Dilworth, 1966). Unless otherwise stated, samples (5 ml) were removed from EPPS-buffered liquid BG-llo cultures and placed into 50 ml Erlenmeyer flasks. The flasks were sealed with rubber stoppers and 5 ml of acetylene (atmospheric pressure) was added per flask by syringe. Gas samples (0.5 ml) were analyzed for their ethylene content at the beginning and at various intervals during the incubation period with a Shimadzu GC-3BF gas chromatograph.

Nitrogenase Activity in the Dark

When the cultures growing in EPPS-buffered BG-llo medium were at the late log phase (about 2 weeks of culturing), 9 ml samples were transferred to 50 ml Erlenmeyer flasks. The flasks were incubated at 28°C in the dark. After 24 h in the dark, 1.0 ml of 10% glucose, fructose or sucrose was added to different flasks to serve as sole energy source in the dark. For each isolate two flasks were not supplemented with an energy source. One of these two flasks was exposed to light while the other, along with those that had been enriched with sugar, were kept in darkness. All assays for nitrogenase activity commenced 8 h after the energy source was added to some of the flasks.

Gel Electrophoresis of Phosphoglucose Isomerase (PGI, EC:5,3,1,9) and Phosphogluconate Dehydrogenase (PGD, EC:1,1,1,44)

Cultures growing in 0.01M EPPS-buffered BG-llo for about 2 weeks were used for enzyme extraction. A crude enzyme extract was obtained by repeated sonication for 30 seconds at a time until more than 60% of the cells were broken . The cells were kept in an ice bath for about 30 second after each sonication. Horizontal electrophoresis on a gel which consisted of 6% polyacrylamide and 4% starch in a L-histidine-NaOH buffer (0.005M L-histidine solution adjusted to pH 7.0 with 4M NaOH) was carried out using a 0.13M tris-citrate electrode buffer (pH 7.0). The samples were applied to the gel according to the modified procedure described by Chen *et al.* (1989). After electrophoresis, the PGI band in the gel was revealed histochemically by immersing the gel in a solution which contained 10 mg β-nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), 0.5 ml 10% $MgCl_2$, 10 mg 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), 2 mg phenazine methosulfate (PMS), 40 units D-glucose-6-phosphate dehydrogenase (disodium salt), and 60 mg fructose-6-phosphate in 50 ml 0.2 M tris-

HCl buffer, pH 8.0, at room temperature. The PGD band was revealed with a reaction mixture containing 15 mg phosphogluconic acid, 15 mg NADP, 0.5 ml 10% $MgCl_2$, 10 mg MTT and 1 mg PMS in 50 ml 0.2 M tris -HCl buffer (pH 8.0).

α-Ketoglutarate Dehydrogenase Activity

α-Ketoglutarate dehydrogenase was assayed by monitoring the α-ketoglutarate-dependent change in absorbance at 340 nm of β-nicotinamide adenine dinucleotide (NAD^+) according to the method of Fisher and Magasanik (1984). Protein extracts from *E. coli* growing under aerobic and anaerobic conditions respectively, were used as controls (Amarasingham and Davis, 1965). A unit of enzyme is defined as the amount causing an increase in optical density at 340 nm of 0.001 per minute. Protein was determined by the Lowry method (Lowry *et al.*, 1951), using crystalline bovine serum albumin as standard.

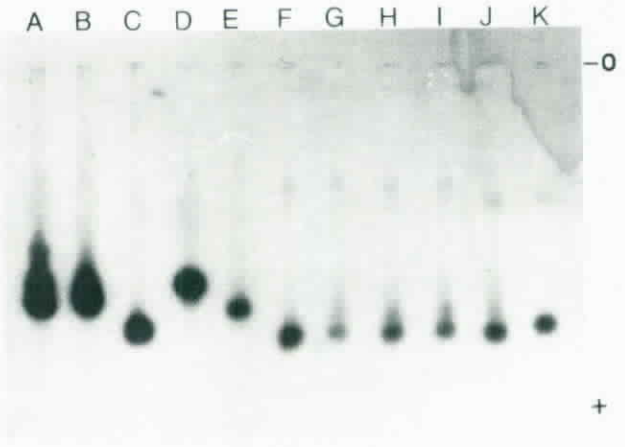

Fig. 1. Zymograms of phosphoglucose isomerase (PGI) from different *Calothrix* isolates. The PGI was extrated by sonication and analyzed qualitatively by 6% polyacrylamide-4% starch continuous gel-electrophoresis. A: HC101; B: HC205; C: HC301; D: HC401; E: HC601; F: HC605; G: HC610; H: HC615; I: HC620; J: HC701; K: HC705.

Results

The phosphoglucose isomerase (PGI) and phosphogluconate dehydrogenase (PGD) extracted from the *Calothrix* isolates usually were stable and could be detected by histochemical staining after gel electrophoresis (Fig. 1). No isozymes were found for the PGI or PGD extracted from the isolates, except for *Calothrix* HC101 which contained two PGI activity bands. Among the isolates, *Calothrix* HC101, HC205, HC301, HC401, HC601, HC605 and HC701 could be differenciated by the relative electrophoretic mobility of their PGI and PGD as analyzed by gel electrophoresis (Table 1). Consequently these seven isolates were selected and characterized.

The morphological features of the heterocystous filaments and hormogonia of the seven isolates, when growing in 0.01M EPPS-buffered BG-llo, is summarized in Table 2. Based on the morphology of the basal trichome region during early development (Fig. 2), the isolates can be grouped into two types: *Calothrix* HC101, HC205, HC301 and HC401 whose basal trichome region do not enlarge significantly during filament development are referred to as "cylindrical types", whereas *Calothrix* HC601, HC605 and HC701 whose basal trichome region enlarged substantially during filament development are referred to as "coryneform types". As shown in Table 2, the hormogonia of the coryneform types are quite similar, all consist of relatively short trichomes with tapering ends and the cells are not vacuolated. The hormogonia of the cylindrical types do, however, differ from isolate to isolate. The hormogonia of HC101 and HC205 are more

Table 1. *Relative electrophoretic mobility (Rf) of phosphoglucose isomerase (PGI) and phosphogluconate dehydrogenase (PGD) extracted from different* ***Calothrix*** *isolates. The crude enzymes were extracted by sonication from different* ***Calothrix*** *isolates and analyzed by continuous polyacrylamide (6%)-starch (4%) gel electrophoresis*

Enzyme	Rf Value						
	HC101	HC205	HC301	HC401	HC601	HC605	HC701
PGI	0.357[a]	0.357	0.397	0.334	0.370	0.40	0.40
PGD	0.258	0.223	ND[b]	ND	0.134	0.178	0.196

[a]There were two PGI activity bands for *Calothrix* HC101. The Rf value indicated is for the faster of the two bands.
[b]ND: enzyme activity was not detected in the gel after electrophoresis.

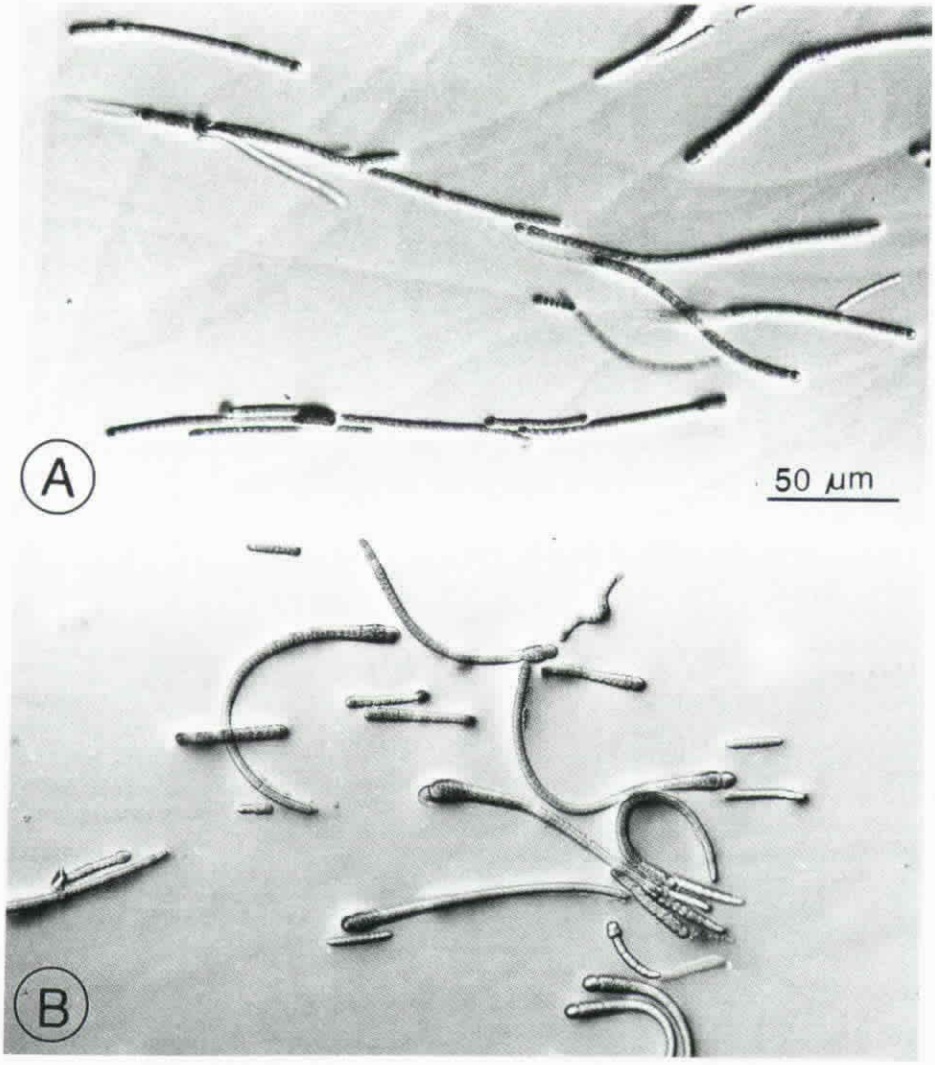

Fig. 2. Comparison of the trichrome morphology of *Calothrix* HC205 (cylindrical type) and HC605 (coryneform type) after 2 weeks on BG-11o agar. A: HC205; B: HC605. The same magnification was used in both cases.

vacuolated and relatively longer than the hormogonia of HC301 and HC401.

The hormogonia of *Calothrix* HC605, one of the coryneform types, were found to adhere to one another tightly during the early developmental stage. The hormogonia twine around one another to form a rope-like structure (Fig. 3). This close-contact "rope-forming" phenomenon has hitherto not been observed in cultures of any of the other isolates although their hormogonia might move in groups (data not shown).

As shown in Table 3, the cylindrical types were more tolerant to acidic conditions than the coryneform types. Among the cylindrical types, *Calothrix* HC101 grew very well in 0.01M MES-buffered BG-11o even at pH 5.5. All seven *Calothrix* isolates grew chemoheterotrophically in 0.01M EPPS-buffered BG-11o, pH 8.0, supplemented with either glucose or fructose. As shown in Table 3, some of them also could use I-inositol, D-ribose, sucrose or D-xylose, as sole energy source to support their dark heterotrophic growth. However, none of the isolates grew in darkness in EPPS-buffered BG-11o medium supplemented with D-arabinose, lactose, D-mannitol, L-rhamnose, sodium acetate, sodium citrate or sodium succinate (data not shown).

When *Calothrix*, grown in EPPS-buffered BG-11o

Table 2. *The morphological characters of the* ***Calothrix*** *isolates growing in BG-llo medium, supplemented with 0.01M EPPS, pH 8.0*

Isolates	Trichome basal region	Heterocystous filaments			Hormogonia	Color
		Vegetative cells	Heterocysts	Akinetes		
HC101	Does not enlarge significantly during filament development.	Rod (5×7 μm)	Rounded (4.5 μm) or oval (4.5×6 μm)	Present (single or serial)	Narrower than heterocystous filaments, relatively long (usually more than 20 cells), slightly vacuolated.	Blackish blue
HC205	Does not enlarge significantly during filament development.	Short rod or barrel-shaped (4.5×5 μm)	Rounded (4.5 μm) or oval (4.5×6 μm)	Present (single or serial)	Narrower than heterocystous filaments (about 3 μm wide), relatively long, highly vacuolated.	Brown
HC301	Does not enlarge significantly during filament development.	Short rod or barrel-shaped	Rounded (4.5 μm)	Present (many in old culture)	Short filaments (usually containing about 5-6 cells), not vacuolated.	Yellowish green
HC401	Does not enlarge significantly during filament development.	Rod (4×6 μm)	Rounded (4.5 μm) or oval (4.5×6 μm)	Present (basal region next to heterocyst)	Short filaments, not vacuolated.	Brown
HC601	Enlarges during filament development.	Cells as long as wide or shorter.	Spade-shaped or hemispherical.	Not seen	Short filaments, tapering at both ends, not vacuolated.	Green
HC605	Enlarges during filament development.	Cells as long as wide or shorter.	Spade-shaped or hemispherical.	Not seen	Short filaments, tapering at both ends, not vacuolated.	Blackish blue
HC701	Enlarges during filament development.	Cells as long as wide or shorter.	Spade-shaped or hemispherical.	Not seen	Short filaments, tapering at both ends, not vacuolated.	Blackish blue

Table 3. *Comparison of some physiological properties of the* ***Calothrix*** *isolates*

Isolates	Growth in acidic BG-llo[a]			Dark heterotrophic growth using[b]					
	pH6.5	pH6.0	pH5.5	Glu	Fru	Ino	Rib	Suc	Xyl
Cylindrical types:									
HC101	+++	+++	+++	+(8 days)[c]	+	−	+	+	+
HC205	+++	+++	+	+(6 days)	+	+	−	+	+
HC301	+++	+++	+	+(10 days)	+	+	+	−	−
HC401	+++	++	±	+(2 weeks)	+	+	−	−	+
Coryneform types:									
HC601	±	±	−	+(2 weeks)	+	−	−	−	+
HC605	+	±	−	+(10 days)	+	−	−	−	−
HC701	+	±	−	+(4 weeks)	+	−	+	+	−

[a] Growth was compared after 3 weeks of culturing. The BG-llo, supplemented with 0.01M MES (2 [N-morpholino] ethanesulfonic acid), was adjusted to pH6.5, pH6.0 or pH5.5 with 1 N HCl.
[b] Glu= Glucose; Fru=Fructose; Ino=I-Inositol; Rib=Ribose; Suc=Sucrose; Xyl=D-Xylose.
[c] The approximate generation time.
+: Growth; −: Death; ±: The cells survived, but no growth.

without an energy source, was shifted from light to dark, its nitrogenase activity decreased slowly to zero within about 15 h. If the culture was shifted back to light or was provided with a suitable organic energy source in the dark, the nitrogenase activity immediately reappeared and gradually attained a constant rate within 5 h (data not shown). The relative nitrogenase activity of the seven *Calothrix* isolates, when provided with glucose, fructose or sucrose as sole energy source in the dark, is listed in Table 4. In the presence of glu-

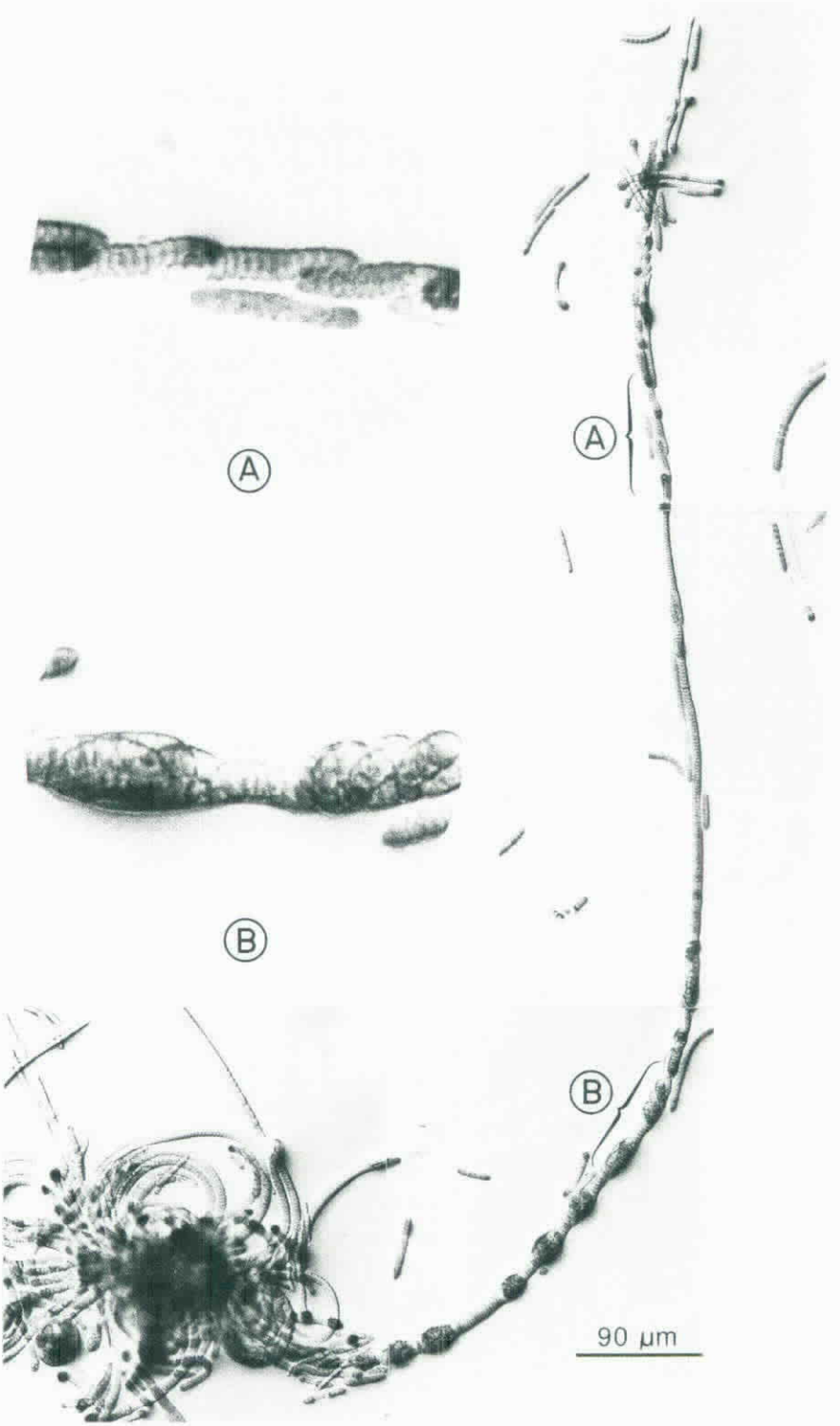

Fig. 3. The close-contact rope-forming phenomenon of the hormogonia of *Calothrix* HC605. The photographs were taken after the trichrome fragments were grown on BG-11o agar for one week. Two portions (indicated by brackets) were enlarged an additional 3.75 times as indicated in insets A and B.

Table 4. *Comparison of the nitrogenase activity of* ***Calothrix*** *isolates under photoautotrophic and dark heterotrophic conditions. The cultures were starved for 24 h by keeping them in the dark whereafter they were either provided with sugar (1.0%) or exposed to light (3000 lux). Nitrogenase activity assays commenced 8 h after the end of the dark starvation period*

Strains	Nitrogenase activity [nmol $C_2H_4 \cdot (\mu g\ chl a)^{-1} \cdot h^{-1}$]			
		Darkness		
	Light	Glucose	Fructose	Sucrose
Cylindrical types:				
HC101	1.3	2.1(160%)[a]	0.3(23%)	0.1(8%)
HC205	2.5	1.3(52%)	1.6(64%)	1.0(40%)
HC301	1.4	0.8(57%)	0.6(43%)	0.2(14%)
HC401	1.7	1.6(94%)	1.2(70%)	0.2(12%)
Coryneform types:				
HC601	2.5	1.0(40%)	0.1(4%)	0.1(4%)
HC605	4.0	1.0(25%)	0.2(5%)	0.1(3%)
HC701	3.0	0.8(27%)	0.1(3%)	0.1(3%)

[a] The nitrogenase activity in the dark expressed as a percentage of the activity in the light.

Table 5. *Effect of nitrate and/or glucose on the nitrogenase activity of* ***Calothrix*** *isolates growing in light*

Strains	Nitrogenase activity [nmol C_2H_4 $(\mu g\ chl a)^{-1}h^{-1}$]		
	BG-llo	BG-llo +0.1% Nitrate	BG-llo +0.1% Nitrate 1% Glucose
Cylindrical types:			
HC101	6.5	0	0
HC205	2.5	0	0
HC301	3.6	0	0
HC401	5.7	0	0
Coryneform types:			
HC601	7.0	0	0.8
HC605	8.8	0	0.6
HC701	12.7	0	1.2

cose or fructose, the cylindrical types in most cases had a dark nitrogen-fixing activity that was more than 50% of its nitrogen-fixing activity in the light. Except for isolate HC101, the cylindrical types had similar nitrogen-fixing activities whether glucose or fructose was used as sole energy source. For the coryneform types, however, the dark nitrogen-fixing activity in the presence of glucose or fructose was never higher than 40% of their activity in the light. In the dark the activity also was much higher in the presence of glucose than in the presence of fructose as sole energy source.

α-Ketoglutarate dehydrogenase activity could not be detected in any of the seven isolates when they were grown in the light or the dark in 0.01M EPPS-buffered BG-llo supplemented with 0.5% glucose. When the isolates were grown in 0.01M EPPS-buffered BG-llo supplemented with 0.5% glucose plus 0.1% sodium acetate, no α-ketoglutarate dehydrogenase activity could be detected in any of the isolates except HC401 which showed weak activity (about 2 units/mg protein).

The addition of 0.1% sodium nitrate to the BG-llo medium completely repressed the N_2-fixing activity of all the *Calothrix* isolates (Table 5), but in the presence of 1% glucose about 10% of the N_2-fixing activity of the coryneform types remained despite the presence of the nitrate. The N_2-fixing activity in the glucose-nitrate-BG-llo medium did not change even after the medium was renewed 4 times by subculturing at one week intervals. A similar antagonistic effect of glucose on the repression of N_2-fixation by nitrate was not observed with the cylindrical types.

Discussion

In addition to differences in their morphology, differences in the physiological or biochemical properties of micro-algae could be very useful in their classification (Kessler, 1978). Laboratory-grown axenic *Calothrix* isolates usually are difficult to identify solely on a morphological basis. Gel electrophoresis, which enables one to compare different proteins in terms of size and/or charge is a very powerful technique. It is also a rapid method requiring only small amounts of material. A comparison of the zymograms of phosphoglucose isomerase, phosphogluconate dehydrogenase, isocitrate dehydrogenase or superoxide dismutase which had been obtained by means of gel electrophoresis has been shown to be useful for the identification of *Anabaena* strains (Glaszmann and Roger, 1985). The zymograms of 6-phosphogluconate dehydrogenase (PGD) and phosphoglucose isomerase (PGI) of *Calothrix* were examined by gel electrophoresis during this study. It was found that the PGI and PGD of *Calothrix*, extracted by sonication, were stable and could be detected by histochemical staining after

gel-electrophoresis. The results also show that the *Calothrix* isolates can be distinguished from one another by the relative electrophoretic mobilities of their PGI and PGD during gel-electrophoresis. Therefore, gel electrophoresis seems to have a great potential value in the identification of micro-algae.

It is known that the basal region of the trichomes of some *Calothrix* taxons enlarges substantially during development. This enlargement, along with other morphological aspects of the trichromes such as the presence or absence of akinetes or hairs, have been used as major distinguishing characters for the classification of *Calothrix* at species level (Desikachary, 1959). In this study, the *Calothrix* isolates were grouped into coryneform and cylindrical types based on the character of their trichome's basal region. The results also indicate that the coryneform and cylindical types differ with respect to some of these physiological properties, such as (a) tolerance to low pH, (b) the relative ability to use glucose or fructose as sole energy source for dark nitrogen-fixation and (c) the antagonistic effect of glucose on the repression of nitrogen-fixation by nitrate. This seem to suggest that the morphological difference of the basal trichome region is a useful character for distinguishing two physiologically different *Calothrix* groups.

Although some species of *Anabaena*, *Nostoc* and other genera are capable of dark heterotrophic growth, many cyanobacteria are obligate autotrophs. All seven *Calothrix* isolates tested in this study are capable of growing in the dark. All eleven *Calothrix* taxons studied by Rippka *et al.* (1979) were capable of growing photoheterotrophically. *Calothrix brevissima* and *C. membranacea* (Khoja and Whitton, 1971) and the endosymbiotic *Calothrix* isolated from a cycad (Huang and Grobbelaar, 1989) have also been reported to be able to grow heterotrophically in the dark. It would therefore appear that the potential to grow heterotrophically in the dark is more common amongst *Calothrix* species than for cyanobacteria in general. The mechanism whereby cyanobacteria can grow heterotrophically in the dark is unknown. It has been suggested that the absence of a complete TCA cycle, especially the absence of α-ketoglutarate dehydrogenase and NADH oxidase, might be responsible for the inability of cyanobacteria to grow heterotrophically in the dark (Smith *et al.*, 1976). However, in the present study it was found that although all the *Calothrix* isolates could grow in darkness, it was only isolate HC401 which exhibited α-ketoglutarate dehydrogenase activity and then only feebly under certain cultural conditions. The results therefore show that α-ketoglutarate dehydrogenase is not a prerequisite in the case of *Calothrix* spp. for the potential to grow heterotrophically in the dark.

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眉藻水田分離株之特性

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分離自本省水田之各種眉藻，利用他們之 phosphoglucose isomerase 和 phosphogluconate dehydrogenase 在膠體電泳時相對泳動速度之不同，篩選出 7 藻株。根據藻絲在發生過程中之基部形態，藻株 HC101、HC205、HC301 和 HC401 歸屬於〝圓柱形〞(cylindrical type)，而 HC601、HC605 和 HC701 等歸屬於〝棍棒型〞(coryneform type)，並對這些藻株的形態與生理性質加以比較。他們雖然都可行異營生長，卻均缺乏或僅有極弱之 α-ketoglutarate dehydrogenase 活性。0.1%硝酸鈉可完全抑制其固氮活性，但屬於棍棒型之三藻株，如在培養基內加入 0.5%葡萄糖，則可使此硝酸抑制作用部份開釋，但對於圓柱形之藻株，葡萄糖並無開釋硝酸抑制作用之效果。本研究並發現藻株 HC605 在發生過程中，其同型體有緊密結合在一起之現象。