THE PHYSIOLOGY OF N_2 FIXATION BY NON-HETEROCYSTOUS CYANOBACTERIA

INDIVIDUAL PROJECT WORK - MICROBIAL DIVERSITY COURSE 1995

GRAZYNA E. SROGA
Molecular Genetics and Cell Biology
University of Chicago, CLSC
920 East 58 Street, Chicago, IL 60 637

INTRODUCTION

Restricted to procaryotes in general, the ability to fix dinitrogen (diazotrophy) is universal amoung those cyanobacteria that produce heterocysts. It is much less widespread among non-heterocystous cyanobacteria. Most species of non-heterocystous cyanobacteria examined so far fix N_2 only during the dark phase of a dark-light cycle and some additionally require microaerobic conditions. Among unicellular cyanobacteria *Gloeothece* spp., *Synechococcus* SF-1, *Cyanothece*, *Aphanothece* and *Synechocystis* were reported to fix N_2. In a case of filamentous non-heterocystous cyanobacteria, aerobic N2 fixation has been reported in several strains of *Oscillatoria* including *Trichodesmium* spp. and *Microcoleus chthonoplastes*. However, *Trichodesmium* species differ substantially from all other non-hetreocystous N_2 fixers. They exhibit light-dependent N_2 fixation in highly oxygenated environments.

Most studies on N_2 fixation by non-heterocystous cyanobacteria have centered on those organisms that can fix N_2 aerobically. One reason for this particular interest in aerobes is that a current goal of agricurtural research is to transfer functional N_2 fixation (*nif*) genes to the chloroplasts of higher plants, where ATP and reductant are readily available. It is widely considered that cyanobacteria resemble the evolutionary ancestors of contemporary chloroplasts, so study of aerobic N_2 fixation in non-heterocystous, especially unicellular cyanobacteria seems to be the most relevant.

Nitrogenase (N_2ase) is a complex enzyme with remarkably similar properties in about 25 organisms in which it has been investigated. It consists of 2 iron-sulphur proteins, that individually, have no detectable enzymatic activity. Together they catalyse the reduction of variety of substrates, i.e. N_2, N_3^-, N_2O, HCN, C_2H_2, CH_3NC or H_3O^+. The lack of the strict specificity towards N_2 allowed development of a relatively strightforward assay of N_2ase activity: the acetylene reduction assay combined with the use

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of gas chromatography. Additional advantage of the method is the fact of using intact (i.e. fully viable) cells in the assay.

The primary goal of the project is to test some features of a cyanobacterial strain classified as *Microcoleus* spp.. Preliminary tests of N_2 fixation (done at the lab from which I got the strain, Amsterdam, The Netherlands) suggest that the strain fixes N_2 in a similar way as *Trichodesmium* . In order to do the N_2 fixation assay on *Microcoleus* spp. and compare it with a few chosen cyanobacterial strains, some additional experiments need to be done:

1 - *Microcoleus* spp. is not axenic.

Learning of purification procedures of cyanobacterial strains from a natural environment (the Sippewissett Salt Marsh, Woods Hole, Mass.) at the course is applied for the purification of *Microcoleus* spp..

2 - *Microcoleus* spp. grows in the form of big, compact aggregates, which are not disrupted by vortexing. From the literature data it is known, for example, that in order to get fine suspension cultures of higher plants, a commercially available composition of cellulolytic enzymes is added to the medium. Since it is not known which enzymes can weaken *Microcoleus* spp. sheaths and cell walls, an attempt to find a bacterial strain capable of digesting cell walls of *Microcoleus* spp. will be made. However, a mixture of a few cellulolytic enzymes will also be made and tested.

3 - Assay of N_2 fixation by acetylene reduction.

The following strains will be tested first: *Synechococcus* WH 8902, *Synechococcus* WH 8904 and *Trichodesmium* NC. The *Microcoleus* spp. strain will be tested after purification. Then the N_2 fixation pattern of *Microcoleus* spp. will be compared with the ones of the above strains.

4 - The strain was named *Microcoleus* based on its morphology. Sequence comparison of some regions of 16S rRNA of such cyanobacteria as *Microcoleus chthonoplastes*, *Lyngbya, Oscillatoria* and *Synechococcus* WH 8904 with the respective sequence of *Microcoleus* spp. should reveal the identity of *Microcoleus* spp. The sequencing data can be used for other studies.

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MATERIALS AND METHODS

1. PURIFICATION OF CYANOBACTERIAL STRAINS.

Samples of cyanobacterial mat were collected at the Sippewissett Salt Marsh, Woods Hole, Mass.. Pieces of the matt containing cyanobacteria were placed on the 2 x SNAX solid medium. Plates were checked every week and some filaments were transferred in a sterile manner onto the fresh solid 2 x SNAX medium.

Microcoleus spp. aggregates of 0.5 to 1.0 cm in size were placed on the 2 x SNAX solid medium. Every week filaments that glided away from the aggregate were picked up with the sterile flat niddle under the binocular microscope and transferred onto the fresh solid 2 x SNAX medium.

The composition of 2 x SNAX: 20 uM Na_2CO_3, 2.0 mM $NaNO_3$, 200 uM NH_4Cl, 20 uM K_2HPO_4, 0.2 ml Cyano Trace Metals, 3.0 uM EDTA, 750 ml filtred Sea Water, 250 ml double-distilled water.

2. MAKING A FINE SUSPENSION OF *MICROCOLEUS* SPP.

2.1. BACTERIAL STRAIN ISOLATION

Isolation of *Myxobacteria* was done during the course classes (Peterson, 1969).

2.2. TEST OF A CELLULOLYTIC ENZYMES MIXTURE

Three enzymes were used: Beta-glucuronidase from *Helix pomatia* type H1 (Sigma), Cellulase E.C. 3.2.1.4 from *Aspergillus niger* (Sigma) and Chitinase E.C. 3.2.1.14 from *Serratia marcescens*. The concentration of each enzyme in the stock solution was 0.05 g/ml. 100 ul and 250 ul of the stock was added per 50 ml of the *Microcoleus* spp. culture, respectively. Cultures were incubated in a growth chamber with 14-hour light and 10-hour dark cycle at 25 C.

3. ASSAY OF N_2 FIXATION BY ACETYLENE REDUCTION

20 ml of cyanobacterial culture was used as an inoculum for 100 ml of the liquid 2 X SO medium (2 x SNAX medium without $NaNO_3$ and NH_4Cl). After 7 days 50 ml of the above culture was inoculated to 0.5 l of 2 X SO. The flasks were incubated at 25 C in a

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growth chamber with 14-hour light and 10-hour dark cycle for 3 days. Then the measurements were done as follows: first 24 hours 20 ml samples were taken every 6 hours (i.e. 12:00, 18:00, 24:00, 6:00, etc.). When the period of N2 fixation was found, assays were done every hour starting at 3:00 am till 9:00 am.

Each 20 ml sample was placed into 60 ml bottle and closed with a rubber plug. 100% acetylene was injected through the plug to the final concentration 10% , so the gaseous phase above the sample was the mixture of acetylene and the air. 300 ul of the gaseous phase was taken at time 0 min. (control), 20 min., and 40 min.. Samples of 200 ul were applied to the POROPAK Q column of the SHIMADZU GC-14A gas chromatograph.

4. DETERMINATION OF Chlorophyl a

After the acetylene reduction assay had been completed, each 20 ml sample was filtrated through the GF-4 glass fiber filter on the Millipore filtration system and frosen at -20C. Samples will be used for the chlorophyl a determination (next week) according the following procedure: each pellet will be grinded and extracted twice with 90% (V/V) methanol for 1 hr at 4C in dim light followed by centrifugation at 10.000 g for 10 min. at 4C. The chlorophyl a content is calculated from the absorbance of the methanolic extract at 665 nm using equation:

$$c\ [ug/ml] = OD_{665\ nm} \times 13.9$$

(Tandeau de Marsac and Houmard, 1988)

5. DNA EXTRACTION FROM CYANOBACTERIA (acc. A. Sghir, J. Dore, unpublished)

Cyanobacterial pellet from the 50 ml culture was digested in a resuspension buffer with Lysosyme, Pronase, Mutanolysin and RNase at 37 C for 1 hr.Then the extraction buffer was added and the incubation was continued for 1 hr at 37 C and for 30 min. at 55 C. DNA was extracted with phenol, chloroform and isoamyl alkohol (totally 6 consecutive extractions).Final aqueous phase was used for DNA precipitation. Precipitated DNA was stored at 4 C untill the use.

6. MICRO PCR FOR rDNA-PCR OPTIMIZATION

The universal 16S rRNA primers were used. PCR was done in the standard PCR buffer. The volume of the reaction mix was 12.5 ul. PCR was initiated by so called "hot-start" procedure.

7. GEL ELECTROPHORESIS

PCR products were analysed by standard gel electrophoresis in agarose.

RESULTS AND DISCUSSION

1. PURIFICATION OF *MICROCOLEUS CHTHONOPLASTES* FROM THE SIPPEWISSETT SALT MARSH AND *MICROCOLEUS* SPP.

Both strains were obtained as the pure cultures (Figure 1 and 2).

The *Microcoleus* spp. was free of bacterial contamination, however some additional tests should be done in order to be sure that the strain is axenic. The amount of the *Microcoleus* spp. is too little in order to start the planned studies. However, the strain is growing very well on the solid medium and soon can be used for propagation in the liquid medium.

The *Microcoleus chthonoplastes* cultures still have bacterial contaminants. The attempt to make them axenic will be done next week.

2. SUSPENSION CULTURES OF *MICROCOLEUS* SPP.

2.1. BACTERIAL STRAINS ISOLATION

Some *Myxobacteria* were isolated. Since the time of the course has been finished, I have no time to test their ability to digest *Microcoleus* spp. cell walls.

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2.2. CELLULOLYTIC ENZYMES

Addition of a mixture of 3 cellulolytic enzymes to the *Microcoleus* spp. medium did not loosed the cell wall structure. The strain was continuing good growth in a form of big aggregates.

3. ASSAY OF N_2 FIXATION

The assay was completed for 2 unicellular marine cyanobacterial strains: *Synechococcus* WH 8902 and WH 8904. Both strains fix N2 at THE END of the dark phase of 10-hour dark and 14-hour light cycle. In the case of *Synechococcus* WH 8902, N_2 fixation still rised during the first hour of of light period and then slowly decresed (Table 1). In the case of *Synechococcus* WH 8904, the decrese of N_2 fixation was already observed during the first hour of light (Table 2).

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Table 1. The pattern of the acetylene reduction of *Synechococcus* WH 8902
(the Light/Dark cycle was 12 hr L (from 6:00 am) / 10 hr D (from 8:00 pm))

Day	Time	Hours passed from the first measurement	Area of the C_2H_4 peak after 20 min.	40 min.
07 14	12:00 noon	0	4	23
	6:00 pm	6	4	5
	12:00 midnight	12	8	3
07 15	6:00 am	18	158	574
	12:00 noon	24	0	0
	8:00 pm	32	0	0
07 16	5:30 am	41.5	498	1746
	7:00 am	43	402	2475
	8:00 am	44	222	1508
07 17	9:00 pm	69	0	0
	12:00 midnight	72	3	0
07 18	3:00 am	75	33	157
	4:00 am	76	185	702

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Table 2. The pattern of the acetylene reduction of *Synechococcus* WH 8904
(the Light/Dark cycle was 12 hr L (from 6:00 am) / 10 hr D (from 8:00 pm)

Day	Time	Hours passed from the first measurement	Area of the C_2H_4 peak after	
			20 min.	40 min.
07 14	12:00 noon	0	0	0
	6:00 pm	6	0	0
	12:00 midnight	12	0	0
07 15	6:00 am	18	836	3538
	12:00 noon	24	36	53
	8:00 pm	32	21	0
07 16	5:30 am	41.5	606	1886
	7:00 am	43	601	1860
	8:00 am	44	173	431
07 17	9:00 pm	69	29	20
	12:00 midnight	72	0	1
07 18	3:00 am	75	195	796
	4:00 am	76	238	997

4. AMPLIFICATION OF 16S rRNA

1.5 kb fragment was amplified by PCR (Figure 3).

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REFERENCES

Peterson J., 1969. Isolation, cultivation and maintenance of the myxobacteria. In: Norris J. and Ribbons D. (eds.). Methods in Microbiology, vol. 3 B , pp. 185-210. Academic Press.

Tandeau de Marsac N. and J. Houmard. Complementary chromatic adaptation: Physiological conditions and action spectra. In: Packer L. and Glazer A. N. (eds.). Methods in Enzymology, vol. 167, p. 322.Academic Press.

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