THE PRESENCE AND ABSENCE OF MAGIC SPOT NUCLEOTIDE MODULATION IN CYANOBACTERIA UNDERGOING NUTRITIONAL SHIFT-DOWN

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1. Introduction

The magic-spot (MS) nucleotides, guanosine tetraphosphate (ppGpp; MSI) and guanosine pentaphosphate (pppGpp; MSII) have been shown to be associated with the regulation of stable RNA synthesis in *Escherichia coli* [1–3]. A rapid increase in MSI and MSII can be produced by a shift from rich to poor nutritional conditions [4–6], or by starvation of a stringent auxotroph for a required amino acid [1–3]. Similar results have been obtained with other heterotrophic [7] and photosynthetic bacteria [8], and with the photoautotrophic cyanobacterium *Anacystis nidulans* [9]. The modulation of MS nucleotides in all prokaryotes examined has led to extensive discussion as to the means by which these compounds are involved in the control of stable RNA synthesis [10]. This paper documents the absence of MS nucleotide variation from several species of cyanobacteria under conditions in which stable RNA synthesis was reduced, and we suggest this excludes these nucleotides from having a universal role in the control of stable RNA synthesis in prokaryotes.

2. Materials and methods

Poly(AGU), GTP and ATP were obtained from Boehringer Mannheim GmbH, Germany, DNAase (RNAase-free) from BDH Chemicals Ltd, England. [α-^{32}P]GTP, [^{32}P]orthophosphate and [2-^{14}C]uracil were obtained from the Radiochemical Centre, Amersham, England. PEI-Cellulose thin layer plates were obtained from Schleicher and Schüll, Dassel, FRG. *Escherichia coli* tRNA was a gift from Dr D. S. Jones.

2.1. *Organisms and growth conditions*

Anacystis nidulans (The Culture Collection of Algae at the University of Texas, Austin, Texas, UTEX 625); *Aphanocapsa* 6308 (supplied by Dr A. J. Smith, Department of Biochemistry, University of Wales, Aberystwyth, the number referring to Professor R. Y. Stanier's collection; this organism was previously classified as *Gloeocapsa alpicola*); *Aphanocapsa* 6714 (supplied by Professor Stanier, Institut Pasteur, Paris); *Nostoc* sp. (known also as *Nostoc* MAC, supplied by Dr A. J. Smith); *Anabaena catenula* (No. 1403/1, The Culture Collection of Algae and Protozoa, Botany Department, University of Cambridge) were all grown on low phosphate medium C as described previously [9] except that Tris was replaced by Hepes (0.3 g/l, pH 7.5). *Anabaena cylindrica* (Cambridge No. 1403/2a) was grown on the medium of Allen and Arnon [11] modified by the addition of 4 mM NH_4Cl and reduction of KH_2PO_4 concentration from 350 mg/l to 7 mg/l, the medium being buffered with Hepes (0.3 g/l, pH 7.5). *A. nidulans*, *Nostoc* MAC and *Aphanocapsa* 6308 were grown at 34°C and 20 cm from a 75 W reflector lamp. All other species were grown at 30°C, and 20 cm from a 60 W incandescent light bulb. All cultures were gassed with air:carbon dioxide (95:5, v/v). Shift down was achieved by transfer of cultures to total darkness. *E. coli* (MRE 600) was grown at 37°C as described by Monier [12].

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2.2. *Extraction and identification of nucleoside polyphosphates*

Cultures were grown with [^{32}P]orthophosphate (100 μCi/ml) for a minimum of one generation before transfer to the dark. Aliquots were removed at intervals and treated as described previously [9]. Nucleoside polyphosphates were separated on polyethyleneimine (PEI)-cellulose thin-layer plastic or glass plates in the one-dimensional system of Cashel [1] and the two-dimensional system of Cashel and Kalbacher [3]. ^{32}P-Labelled nucleotides were located by autoradiography, cut from the plate and counted in a liquid scintillation counter.

2.3. *Measurement of RNA synthesis*

A. cylindrica was growth with [2-^{14}C]uracil (0.2 μCi/ml, 3 nmol/ml) together with carrier (3 nmol/ml). Samples (1 ml) were removed and added to equal volumes of uracil (4 μmol/ml) and 10% (w/v) trichloroacetic acid, kept at 0–3°C for 30 min, and the precipitates collected on 0.45 μm cellulose nitrate filters. The filters were washed with ice-cold 5% (w/v) trichloroacetic acid (5 ml), followed by 5 ml uracil (4 mM) and 50 ml distilled water. The filters were dried, gummed to planchets and counted in a Spectro/Shield low background planchet counter.

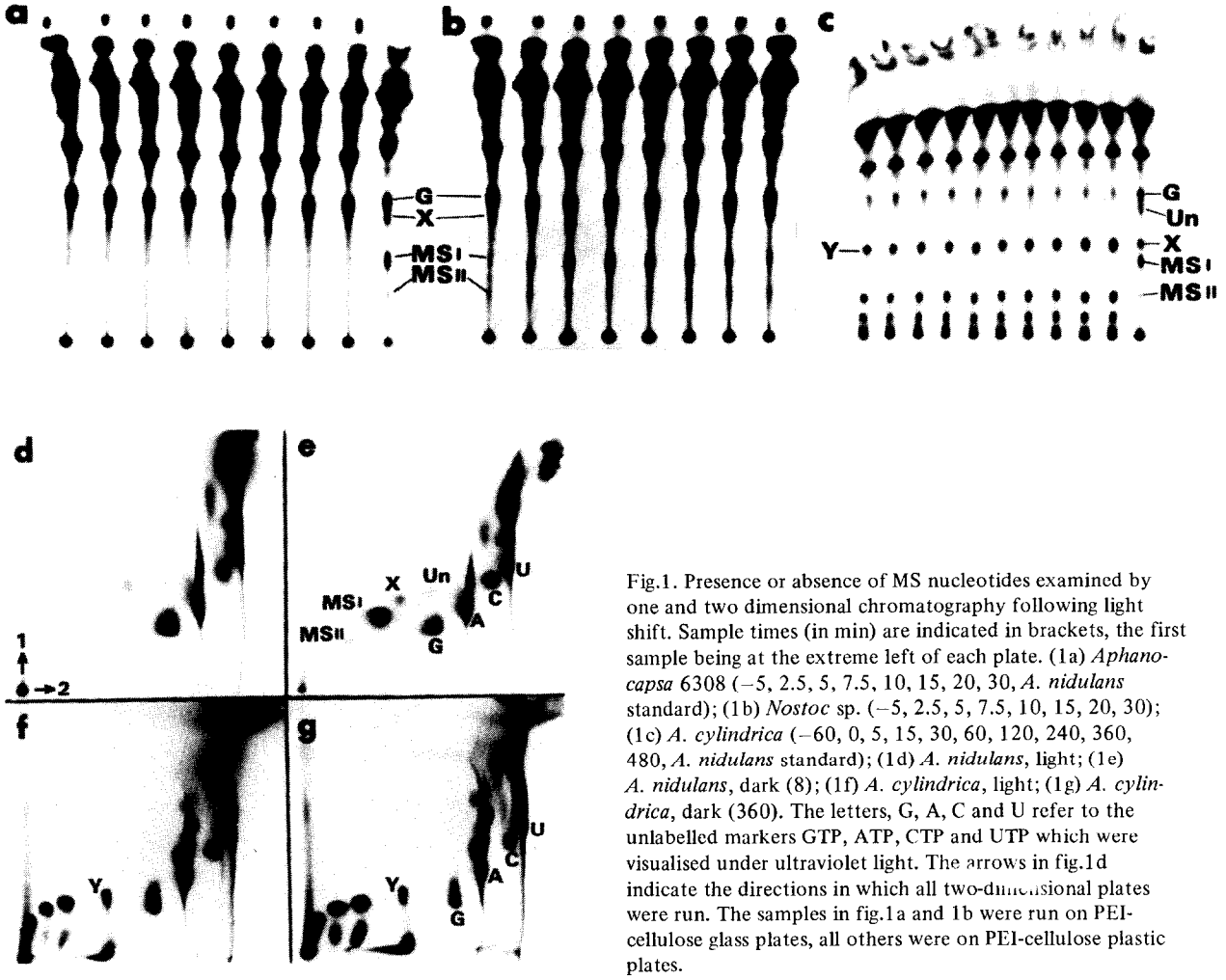

Fig.1. Presence or absence of MS nucleotides examined by one and two dimensional chromatography following light shift. Sample times (in min) are indicated in brackets, the first sample being at the extreme left of each plate. (1a) *Aphanocapsa* 6308 (–5, 2.5, 5, 7.5, 10, 15, 20, 30, *A. nidulans* standard); (1b) *Nostoc* sp. (–5, 2.5, 5, 7.5, 10, 15, 20, 30); (1c) *A. cylindrica* (–60, 0, 5, 15, 30, 60, 120, 240, 360, 480, *A. nidulans* standard); (1d) *A. nidulans*, light; (1e) *A. nidulans*, dark (8); (1f) *A. cylindrica*, light; (1g) *A. cylindrica*, dark (360). The letters, G, A, C and U refer to the unlabelled markers GTP, ATP, CTP and UTP which were visualised under ultraviolet light. The arrows in fig.1d indicate the directions in which all two-dimensional plates were run. The samples in fig.1a and 1b were run on PEI-cellulose glass plates, all others were on PEI-cellulose plastic plates.

2.4. *Preparation of an in vitro system for MS synthesis*

E. coli (MRE 600) NH_4Cl-washed ribosomes and 0.5 M NH_4Cl ribosomal wash (containing stringent factor) were prepared as described by Haseltine et al. [13]. Ribosomes from *Aphanocapsa* 6308 and *A. nidulans* were prepared by harvesting and washing cells in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M KCl and 0.01 M $MgCl_2$. After breakage in a French press (16 000 psi), cells were treated with DNAase for 30 min at 4°C and Triton X-100 was added to a final concentration of 5% (v/v). The lysate was centrifuged twice at 15 000 × *g* to remove debris. The supernatant was layered over an equal volume of 1 M sucrose and centrifuged at 40 000 rev/min for 2.5 h at 4°C in a Beckman Ty 65 rotor. The ribosomal pellets were washed with buffer and allowed to resuspended in buffer for 16 h at 4°C.

The synthesis of ppGpp and pppGpp was assayed as described by Sy et al. [14] in a total volume of 50 μl for *Aphanocapsa* 6308 ribosomes and 250 μl for the more dilute *A. nidulans* ribosomes. This volume contained 2 μg/μl *E. coli* ribosomal wash and 1 μCi [α-^{32}P]GTP. The reaction was initiated by the addition of 100 μg ribosomes and after incubation at 30°C for 60 min, was terminated by the addition of 2 μl formic acid (Analar, 98%). After centrifugation to remove precipitated material, 5 μl of the supernatant was taken for one-dimensional chromatography on PEI-cellulose as described above. Spots corresponding to ppGpp, pppGpp and GTP were located by autoradiography, cut out and counted.

3. Results and discussion

The presence of MSI and MSII in *Nostoc* sp. (fig.1b) and their absence from *Aphanocapsa* 6308 (fig.1a) following transfer to dark is shown by one-dimensional chromatography on PEI-cellulose glass plates. A ^{32}P-labelled compound (Y) whose concentration does not alter significantly with nutritional shift was detected in *A. cylindrica* and the mobility of this during one-dimensional chromatography on PEI-cellulose plastic plates (fig.1c) was close to the compound X, previously reported to be present in *A. nidulans* [9]. Two-dimensional chromatography distinguished between X and Y and confirmed the

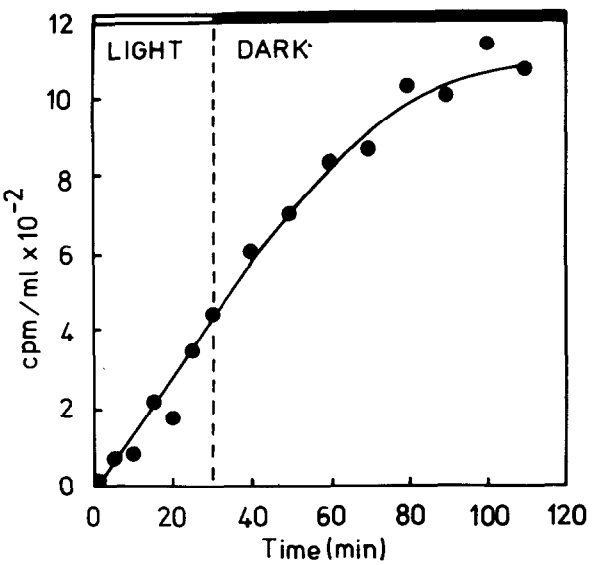

Fig.2. Decline of RNA synthesis in *A. cylindrica* measured by incorporation of [^{14}C]uracil into TCA-precipitable material, following light to dark shift.

presence of the latter in *A. cylindrica* before and after transfer to dark (figs 1f and 1g). No MSI could be detected in *A. cylindrica* before or after shift-down in either chromatographic system (figs 1c,1f and 1g). In contrast, a marked change in MSI concentration can be clearly seen in *A. nidulans* following transfer to the dark (figs 1d and 1e). An unknown phosphorylated compound (Un) separates from GTP in *A. nidulans* standard when run on plastic (fig.1c) but not glass (fig.1a) PEI-cellulose plates. This compound can be seen more clearly following two-dimensional chromatography (figs 1d and 1e).

The incorporation of [^{14}C]uracil into a TCA-precipitable fraction, indicative of RNA synthesis, shows a clear reduction in *A. cylindrica* following transfer to the dark (fig.2). Thus, this organism does not produce detectable amounts of MSI when stable RNA synthesis is reduced. Furthermore, during induction of heterocyst differentiation by removal of ammonia from the medium there was no detectable MSI synthesis for the first 11 h (data not shown).

The variation in MSI, MSII and GTP over a 30 min period following light to dark shift, shows the interrelationship between these compounds in *Nostoc* sp. (fig.3). The compound X is also apparent in this

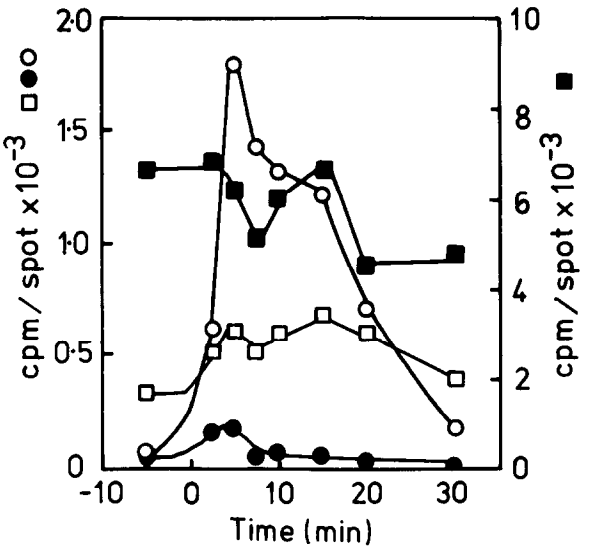

Fig.3. Variation in nucleotide levels in *Nostoc* sp. undergoing shift from light to dark. MSI (○), MSII (●), GTP (■), X (□).

Table 1

Organism	Presence (+) or absence (–) of MS variation following light to dark shift
Anacystis nidulans	+
Nostoc sp.	+
Aphanocapsa 6714	+
Aphanocapsa 6308	–
Anabaena cylindrica	–
Anabaena catenula	–

organism (figs 1b and 3). A restricted survey of six species of cyanobacteria reveals that three clearly vary the concentration of MSI in response to light to dark shift, and that in the other three species MSI is undetectable under either condition (table 1).

Interspecies differences in cyanobacteria with respect to MS production were confirmed using in vitro preparations. It has been shown that chloroplast (but not cytoplasmic) ribosomes from *Chlamydomonas reinhardtii* catalyse MS nucleotide production by *E. coli* stringent factor [14]. Table 2 shows

Table 2
In vitro MS synthesis by cyanobacterial and *E. coli* ribosomes

Additions	Reaction vol. (μl)	%GTP converted to ppGpp + pppGpp
E. coli ribosomes	50	8 (7 – 9) [3]
E. coli ribosomes + *E. coli* ribosomal wash	50	34 (28 –48) [3]
Aphanocapsa 6308 ribosomes	50	1 (0.5– 1.5) [4]
Aphanocapsa 6308 ribosomes + *E. coli* ribosomal wash	50	3 (1 – 5) [4]
E. coli ribosomal wash	50	6 (5 – 7) [4]
E. coli ribosomes	250	1 [1]
E. coli ribosomes + *E. coli* ribosomes wash	250	15 [1]
A. nidulans ribosomes	250	1 (0.5– 1.5) [2]
A. nidulans ribosomes + *E. coli* ribosomal wash	250	14 (12 –16) [2]

Average conversion rates are presented with range ( ) and number of determinations [ ]

that stimulation of MS production from GTP in the presence of *A. nidulans* ribosomes and *E. coli* ribosomal wash, was comparable to that found with the complete *E. coli* system. No such formation of MS nucleotides in vitro was catalysed by *Aphanocapsa* 6308 ribosomes, with or without the addition of *E. coli* ribosomal wash.

Acknowledgements

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