Temporal Genetic Mapping in the Blue-green Alga *Anacystis nidulans* Using Ethyl Methanesulphonate

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SUMMARY

Cultures of the blue-green alga *Anacystis nidulans* were synchronized with respect to DNA synthesis as well as cell division. Application of ethyl methanesulphonate at different stages of replication resulted in a peak of mutation frequency for different genetic markers; this peak can be accounted for in terms of the involvement of repair processes. A temporal map of 19 markers has been constructed by this method. Comparison of gene position obtained by temporal mapping indicates that either bidirectional replication or unidirectional replication from more than one origin occurs.

INTRODUCTION

Although genetic mapping by conventional processes involving gene transfer is possible in a number of prokaryotes, methods for determining gene sequence have been developed which do not depend directly on any gene transfer mechanism. These are based on the estimation of marker frequencies by transformation (Yoshikawa & Sueoka, 1963; Sabatier, Louarn & Sicard, 1972), analysis of density-labelled transducing phage (Caro & Berg, 1968; Nishioka & Eisenstark, 1970), nucleic acid hybridization (Cutler & Evans, 1967; Bird *et al.* 1972), studies on the rates of induced enzyme synthesis (Masters & Pardee, 1965), and mutation induction (Stonehill & Hutchison, 1966; Cerdá-Olmedo, Hanawalt & Guerola, 1968; Vielmetter, Messer & Schütte, 1968). These methods have made possible the construction of genetic maps of organisms with no known mechanism of gene transfer, for example *Streptococcus faecalis* (Stonehill & Hutchison, 1966), and since these techniques depend on replication of the genome they have, in conjunction with conventional genetic mapping, enabled the mode of replication to be determined (Hohlfeld & Vielmetter, 1973).

Asato & Folsome (1970) produced a temporal genetic map of *Anacystis nidulans* for six markers using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) for sequential mutagenesis while Herdman, Faulkner & Carr (1970) located the position of a gene specifying nitrate reductase by u.v. mutagenesis. This communication describes an extension of these studies and the construction of a temporal genetic map of *A. nidulans* for 19 markers. The mutagen was ethyl methanesulphonate (EMS), one which had not been used previously for temporal genetic mapping. Because EMS can readily be inactivated by sodium thiosulphate (Loveless, 1959) and thus the exposure time carefully controlled, its use offers an advantage over many other chemical mutagens whose action can only be terminated either by dilution below an effective level or by harvesting and washing the mutated culture. The latter procedures tend to give inaccurate exposure times.

Table 1. *Mutant strains of Anacystis nidulans*

Strain	Genotype	Phenotype
1114	<i>nitB2</i>	Requires ammonium chloride (7 mM)
1121	<i>cys-1</i>	Requires cysteine (0.5 mM) or sodium thiosulphate (0.1 mM)
1141	<i>bio-1</i>	Requires biotin (2 µg/ml)
1142	<i>bio-2</i>	Requires biotin (2 µg/ml)
1172	<i>met-4</i>	Requires methionine (0.1 mM)
2111	<i>nitA1, str-1</i>	Requires sodium nitrite (2 mM) and is resistant to streptomycin (25 µg/ml)
2113	<i>nitA, met-2</i>	Requires sodium nitrite (2 mM) and methionine (0.1 mM)

METHODS

Organisms. The wild type (0100) was *Anacystis nidulans*, strain No. 625 of the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Indiana, U.S.A. The mutant strains derived from 0100 are described in Table 1. All were revertible by EMS. Stocks of the wild type and mutants were maintained as described by Herdman, Delaney & Carr (1973).

Cyanophage AS-1, lytic against *A. nidulans*, was the gift of Dr R. S. Safferman, Office of Research and Monitoring, Environmental Protection Agency, Cincinnati, Ohio 45226, U.S.A. Stocks were propagated in liquid cultures of the host organism. *Anacystis nidulans* was grown on modified Allen's medium (Herdman *et al.* 1973) in Roux bottles at 34 °C, 8 cm from banks each of three 40 W warm white fluorescent lamps, and was gassed with a mixture of air:CO₂ (95:5, v/v). When the culture density had reached about 10⁸ organisms/ml the culture was infected with 10 ml of phage lysate, which resulted in complete lysis after 24 h. Cell debris was removed by centrifuging at 3000 g for 20 min, and after sterilization by filtration the lysate was stored at 0 °C. Lysates prepared in this way remained active for up to one month. Plaque-forming units were assayed by a modification of the procedure described by Safferman & Morris (1964). To prepare top layers, 2.5 ml of an overnight culture of the host, prewarmed to 48 °C, and 0.1 ml of phage lysate, suitably diluted in medium which had been equilibrated with CO₂, were added to 2.5 ml of 1.2% (w/v) agar (without medium) held molten at 48 °C. Plates were incubated as described by Herdman *et al.* (1973) and scored after 48 h; plaque counts were determined in triplicate.

Growth and induction of synchrony. Synchronized growth was induced by light deprivation as described by Herdman *et al.* (1970). Organisms were grown in a 1 l glass fermentation vessel, immersed in a transparent water bath at 38 °C, illuminated by two 75 W tungsten lamps (Cryselco, Kempton Works, Bedford) at a distance of 14 cm, and gassed with a mixture of air:CO₂ (95:5, v/v). The fermentation vessel was a 1 l flat-bottomed reaction vessel (Quickfit, Gallenkamp, London) fitted with a multi-socket flat flange. When the culture density had reached 5 × 10⁷ organisms/ml, the culture was subjected to a 16 h dark induction period. On return to normal growth conditions synchronized growth was initiated.

Estimation of cell numbers. Total cell numbers were determined using either a Neubauer counting chamber (Hawksley Crystalite, Gallenkamp) or a model ZB Coulter counter (Coulter Electronics, Dunstable, Bedfordshire) with a 70 µm orifice.

Measurement of DNA. DNA was estimated in 20 ml samples from synchronized cultures by the diphenylamine reaction of Burton (1956) modified according to Craig, Leach & Carr (1969).

Sequential mutagenesis. Samples (10 ml) from synchronized cultures were harvested by

aseptic filtration through cellulose nitrate filters (Sartorius, V. A. Howe & Co. Ltd, London) with a pore size of $0.45\ \mu\text{m}$, and suspended in 10 ml of a sterile solution of 0.2 M-EMS in 0.03 M-phosphate buffer pH 7.0. Exposure was at room temperature for 25 min, with the exception of strains 1141 and 1142 which were much more resistant to the mutagen and were exposed for 60 min. Mutagenesis was terminated by the addition of 1 ml sodium thiosulphate (50 %, w/v) after which the organisms were harvested, washed with distilled water and resuspended in 5.0 ml distilled water. The resuspension was inoculated into 50 ml growth medium, supplemented as necessary, in a 250 ml conical flask, and was incubated in an orbital shaker as described by Herdman *et al.* (1973) to permit the segregation of mutant organisms. When the culture density had reached between 1×10^8 and 5×10^8 organisms/ml, the total cell count was determined and samples were plated on selective medium to score for the marker concerned and on propionate-supplemented medium to score for the standard marker, propionate resistance. In most cases samples were plated undiluted. However, mutation to propionate resistance and phage resistance occurred at a higher frequency than usual, and for these, samples were diluted five-fold before plating. Plates were incubated as described by Herdman *et al.* (1973) for 7 days, except where propionate resistance and phage resistance markers were being mapped when plates were scored after 5 days' incubation.

Scoring of mutants. Selection was made either for resistant mutants or for revertants of auxotrophic strains. Mutation frequencies were expressed as the number of mutants/ 10^6 survivors. Yellow, blue and filamentous mutants could not be selected and were scored among the propionate-resistant mutants and the frequencies of these were expressed as mutants/ 10^3 propionate-resistant mutants. It was assumed that scoring mutations in this way did not skew marker positions towards the propionate resistant marker. Phage-resistant mutants were scored by a modification of the cyanophage assay technique described above. Top layers were prepared by adding 2.5 ml of concentrated phage lysate (4×10^6 plaque-forming units/ml), prewarmed to $48\ ^\circ\text{C}$, and 0.1 ml of the segregated culture to 2.5 ml of molten agar. These were poured on to agar plates and after incubation phage resistant colonies could be counted.

Chemicals. Ethyl methanesulphonate and polymyxin B sulphate were obtained from Sigma. Polymyxin was used at $150\ \mu\text{g/ml}$, streptomycin at $25\ \mu\text{g/ml}$ and sodium pyruvate at 5 mM. Concentrations of supplements are given in Table 1.

RESULTS

Growth of synchronized cultures

During the post-induction period all strains examined exhibited a stepwise increase in total cell numbers. The pattern of growth was the same in all strains and was similar to that described by Herdman *et al.* (1970) for the wild type. Synchronized growth of strain 2111 (*nitAI*, *str-I*) is shown in Fig. 1. During synchronized growth, synchronized DNA synthesis (Fig. 2) was observed and this also followed the pattern described by Herdman *et al.* (1970).

Growth rates in synchronized and exponential cultures

Under the same conditions of incubation growth rates were faster during synchronized growth than during exponential growth, as shown in Table 2.

Fig. 1. Increase in total numbers of *Anacystis nidulans* strain 2111 (*nitA1*, *str-1*) following synchronization.

Table 2. Growth rates (doublings/h) of synchronized and exponential cultures of *Anacystis nidulans*

Growth rates of synchronized cultures were determined from the beginning of the first and second division periods.

Strain	Growth rate	
	Exponential	Synchronized
0100 (wild type)	0.37	0.51
1121 (<i>cys-1</i>)	0.26	0.50
1172 (<i>met-4</i>)	0.25	0.50
2111 (<i>nitA1</i> , <i>Str-1</i>)	0.36	0.57

Kinetics of mutation induction during synchronized growth

Sequential mutagenesis with EMS resulted in a peak of mutation frequency (Fig. 2) similar to that observed for NTG in *Escherichia coli* (Cerdá-Olmedo *et al.* 1968) and *Neisseria meningitidis* (Jyssum, 1969). Asato & Folsome (1970) found that NTG produced a peak of mutation frequency in *A. nidulans*, but this was caused by a stepwise increase in mutation frequency which later decreased as the culture divided. However, the peak resulting from EMS mutagenesis cannot be explained in this way since irrespective of whether the absolute number of mutants or the mutation frequency was determined, a peak rather than a stepwise increase was observed (Fig. 2). This suggests that the mutagen acts preferentially at the replication fork (Cerdá-Olmeda *et al.* 1968), the peak corresponding to the replication of the gene. While a peak of mutation frequency was observed, mutagenesis during DNA replication did not detectably alter survival (Fig. 3); thus only the mutagenic effect of EMS and not the lethal effect was enhanced at the replication fork.

Mapping by reference to a standard marker. Since auxotrophic markers were mapped in different strains it was necessary to establish that the pattern of DNA replication was the same in all strains. For this a standard marker, propionate resistance, was mapped in each experiment as a control. This showed a peak in mutation frequency 112 minutes after the initiation of synchronous growth, corresponding to the 37th minute of DNA synthesis (Fig. 2). The constancy of this peak showed not only that the pattern of replication was the same in each strain but also that the DNA synthesis period occupied a constant position in the synchronized growth cycle.

The temporal genetic map of Anacystis nidulans. Sequential mutagenesis of synchronized cultures for the different markers is shown in Figs. 2, 3 and 4. Figure 5 shows the temporal genetic map of *A. nidulans*. Map positions were indicated by peaks in the mutation frequency curves. As a peak represents the mode of a curve rather than the mean, the error on the

Fig. 2

Fig. 2. Induction of propionate-resistant mutants of *Anacystis nidulans* by sequential mutagenesis during DNA replication (□) following synchronization. The first replication cycle occupies the period 75 to 135 min after induction. This is followed by a period of 30 min during which no synthesis occurs, after which a second replication cycle begins at 165 min. ○, Mutants/ 10^6 survivors; ●, mutants/ml.

Fig. 3

Fig. 3. The effect of sequential mutagenesis of a synchronized culture of *Anacystis nidulans* strain 1114 (*nitB2*). (a) Frequency of reversion to wild type of the *nitB2* marker. (b) Synchronous growth (●) and survival after mutagenesis (○). Survival was determined as cell count after the mutated sample had been incubated to permit segregation of revertants.

peak is not open to any conventional statistical analysis. However, since mutation frequency was determined at intervals of no less than 5 min, the error on the map positions may be as great as 5 min. Unlike the previous map of this organism (Asato & Folsome, 1970), the markers span the whole period of the DNA replication cycle. The map positions of the markers *pmbA* (polymyxin B resistance), *yelA* (yellow pigmentation) and *bluA* (blue pigmentation) agree closely with those of similar markers on the map described by Asato & Folsome (1970) as shown in Fig. 5. The position of the *sna* (snake) marker and the *fil* (filamentous) marker do not correspond, although they both exhibit a filamentous phenotype. However, Kunisawa & Cohen-Bazire (1970) have described two types of filamentous mutants of *A. nidulans*, class I and class II: the *Sna* mutants resemble the class I type while all the *Fil* mutants were of the class II type.

Multiple peaks of mutagenesis. For some markers such as *bio-2*, *pmb*, *yel* and *blu* more than one peak of mutation was observed, indicating that mutation at more than one locus could give rise to the same phenotype. Mutations to yellow pigmentation mapped at three sites designated *yelA*, *yelB* and *yelC* (Fig. 4), while mutations to blue pigmentation and polymyxin B resistance mapped at two. Although yellow mutants are frequently defective in the assimilation of nitrate, none of the yellow loci corresponded to either of the genes involved in the reduction of nitrate (*nitA* and *nitB*), suggesting either that there were further

Fig. 4. Sequential mutagenesis of synchronized cultures of *Anacystis nidulans*. Mutation rates were expressed as follows: *met-4*, *bio-1*, *bio-2*, *supA*, *nitAI* (2111), *str*, *pmb* and *aso*, as mutants/ 10^6 survivors; *yel*, *blu* and *fil*, as mutants/ 10^8 propionate-resistant mutants; *cys-1*, *met-2* and *nitAI* (2113), as mutants/ml. Scoring mutations in different ways had no effect on the time at which the peak of mutation occurred.

genes involved in the reduction of nitrate or that these mutants were defective in pigment synthesis.

Mapping of the *bio-2* marker resulted in two types of revertants which mapped at different sites (Fig. 4): large dark colonies resembling the wild type, and small pale colonies. The former were presumed to be true revertants while the latter appeared to be suppressed mutants which mapped at a different site designated *supA*. It was not possible to analyse these genetically to establish whether the original mutation had been suppressed in the latter. Suppressor mutations were not observed when any other auxotrophic markers were mapped.

Mutations to propionate resistance mapped at one site only (*prp*) and this was in agreement with the proposal of Smith & Lucas (1971) that resistance was brought about by mutation in the gene specifying acetate thiokinase. Unless there were several copies of this gene at different sites along the genome, propionate resistance was expected to map at one site only.

Fig. 5. (a) The temporal genetic map of *Anacystis nidulans*. *aso* denotes mutation to cyanophage AS-1 resistance; the other genetic symbols are explained in the text. (b) For comparison, the map obtained by Asato & Folsome (1970), drawn to the same scale, is shown.

DISCUSSION

The increased growth rates of synchronized cultures compared with exponential cultures may have been at the expense of pools of precursors formed during the induction period. Herdman *et al.* (1970) have shown that, in *A. nidulans*, normal cell metabolism continues for a short time after the beginning of the induction period and it is possible that after the cessation of macromolecular synthesis, pools of precursors continue to be built up for some time in the dark.

On the return to normal growth conditions synchronized growth at increased rates may occur at the expense of these pools. Increase in growth rates of synchronized cultures have been observed in *E. coli* (Barner & Cohen, 1956), *Lactobacillus acidophilus* (Burns, 1959) and *Schizosaccharomyces pombe* (Sando, 1963), and have been ascribed to unbalanced growth resulting from synchronization (Mitchison, 1971). Whether synchronized growth of *A. nidulans* was balanced or unbalanced was not considered to be of importance for the purposes of temporal genetic mapping, since it was only necessary for a culture to go through one cycle of synchronized DNA replication irrespective of the state of growth of the organism. However, it must be assumed that the velocity of the replication fork(s) remains constant during this cycle of replication.

Enhanced mutagenesis at the replication point by EMS can be explained in terms of repair processes. In addition to photoreactivation (Asato & Folsome, 1969) there is now good evidence for the existence of dark repair processes in *A. nidulans*. Caffeine and acriflavine are believed to prevent excision of u.v.-induced damage (Setlow, 1964; Clarke, 1967), causing increased sensitivity to the mutagen. Under conditions preventing photoreactivation, increased sensitivity to u.v. light as a result of caffeine treatment (Asato, 1972) and acriflavine treatment (Singh, 1968; Singh, Kumar & Prakash, 1969) of *A. nidulans* has been observed. In addition, a u.v.-sensitive mutant of *A. nidulans* has been shown to be insensitive to the effect of acriflavine (Singh, 1968), indicating that it may lack an excision repair mechanism.

Fig. 6. (a) The temporal genetic map of *Anacystis nidulans*; (b) and (c) represent the conventional genetic map (Herdman, 1973) on which two possible modes of replication can be superimposed. Either (b) the genome is replicated bidirectionally from a single origin, or (c) there is more than one origin and replication proceeds in one direction.

It has been shown that EMS damage in *A. nidulans* cannot be photoreactivated but can be repaired by a dark repair process which is sensitive to caffeine (Delaney & Carr, unpublished data). Any mutational damage induced during sequential mutagenesis of synchronized cultures can presumably be repaired by this process. However, any damage immediately ahead of the replication fork is likely to be replicated before it can be repaired by this repair mechanism. Although the mutation frequency of a gene increased as it was replicated there was no concurrent decrease in survival (Fig. 3), indicating that there was some repair during and immediately after gene replication even though this resulted in enhanced mutagenesis. This situation is analogous to post-replication repair in *E. coli* (Witkin, 1969) where u.v.-induced damage can be repaired after the replication of damaged regions of DNA by a mechanism which tends to introduce mutations. The repair of replicated damage in *A. nidulans* by a similar process would explain enhanced mutagenesis of the replication point without any loss in viability. Once the replication fork has passed, any further damage to a gene could be repaired by the excision repair process before the next round of replication and so the mutation frequency would return to normal. In this way a peak in mutation frequency of a gene would result as that gene was replicated. The possible existence of an error-prone repair process in blue-green algae similar to post-replication repair is supported by the isolation of a mutant which was sensitive to u.v. light but showed reduced mutability (Zhevner & Shestakov, 1972). This mutant resembles the *exr* mutants of *E. coli* which are defective in post-replication repair (Witkin, 1969). Where a mutagen acts preferentially at the replication fork it can be predicted that if a mutation at a particular site is selected for, there will be an increased frequency of mutation in genes adjacent to this site. Thus clusters of closely linked mutations will arise. This has been observed both with NTG (Guerola, Ingraham & Cerdá-Olmedo, 1971) and EMS (Lindegren, Courtis & Schult, 1968).

Transformation in *A. nidulans* mediated by naturally-occurring nucleic acids (Herdman, 1973) has enabled a conventional genetic map for five markers to be constructed. This is shown in Fig. 6. The temporal genetic map can be compared with this map with respect to four markers, *nitA1*, *cys-1*, *met-2* and *bio-1*, on the basis of which two modes of replication can be proposed (Fig. 6). Either replication starts from a single initiation point and is bidirectional, or it is unidirectional and starts from several initiation points. At present it is

not possible to distinguish between these two models, but since bidirectional replication is now well established in other organisms (Huberman & Riggs, 1968; Masters & Broda, 1971; Danna & Nathans, 1972; Gyurasits & Wake, 1973) the former model is favoured.

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