Synthesis of RNA during Myxospore Induction in *Myxococcus xanthus*

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SUMMARY

RNA was isolated from vegetative *Myxococcus xanthus* FB and myxospores. Organisms were labelled with [^{32}P]orthophosphate during encystment and the metabolic stability of the radioactive RNA was followed by fractionating purified RNA from 'pulse-chased' cultures by polyacrylamide gel electrophoresis. Much of the label was in heterodisperse (messenger-like) RNA but the stable RNA (present in the mature myxospores) was largely high-molecular weight ribosomal RNA and its precursors.

The role of RNA synthesis in the morphogenetic cycle of *Myxococcus xanthus* is discussed in the light of these data and of the properties of the ribosomes and ribosomal proteins from this organism.

INTRODUCTION

The stages involved in the induction of myxospore production in *Myxococcus xanthus* are summarized in Fig. 1. The short period of DNA synthesis has been studied by Rosenberg, Katarski & Gottlieb (1967) who concluded that the synthesis corresponded to replication of 20% of the total chromosomes present in the population and that no chromosome division was reinitiated in this period. The nature of the RNA synthesized during the early phases of myxospore induction has been characterized by fractionating the RNA (Bacon & Rosenberg, 1967) and by using RNA:DNA hybridization (Okano, Bacon & Rosenberg, 1972). They concluded that both rRNA (ribosomal RNA) and heterodisperse mRNA (messenger RNA) of lower sedimentation coefficients than 16 S rRNA are synthesized during this period, and that although the rRNA is indistinguishable from that present in vegetatively growing organisms, certain species of mRNA synthesized during myxospore induction are not synthesized during vegetative growth. Conversely, certain species of mRNA synthesized during vegetative growth are absent from organisms undergoing myxospore induction.

At an advanced stage of the process (after refractility had been achieved; see Fig. 1) there is another burst of RNA synthesis (Ramsay & Dworkin, 1970). We refer to this as '4 to 5 h RNA'. Ramsay & Dworkin (1970) isolated this RNA by breaking the organisms with glass beads in a mechanical shaker and fractionated it by sucrose-gradient centrifugation. They compared pulse-labelled RNA with RNA that had been pulsed and 'chased' with actinomycin D. We have extended this study by using different methods for isolating the RNA and fractionation by polyacrylamide-gel electrophoresis. In this paper we compare ^{32}P -pulse-labelled 4 to 5 h RNA with RNA pulse-labelled and 'chased' into mature myxospores in the presence of unlabelled phosphate.

Fig. 1. Stages of myxospore induction in *Myxococcus xanthus* in liquid medium, based on the data of Sadler & Dworkin (1966) and Ramsay & Dworkin (1970).

METHODS

Sources of chemicals were as described by Foster & Parish (1973). Polyvinyl sulphate was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A., sodium triisopropyl-naphthalene sulphonate from Kodak Ltd, Kirkby, Liverpool, precipitated acid-washed silica from BDH, Poole, Dorset and DEAE-cellulose (Whatman, DE23) from H. Reeve Angel Ltd, London.

Myxococcus xanthus FB was grown in shaken cultures of 25 ml in 250 ml conical flasks at 30 °C in an orbital incubator (300 rev./min.) in deionized casitone medium (Foster & Parish, 1973).

Bacillus sp. no. 4 (apparently a strain of *B. licheniformis*) a gift from Professor M. Dworkin, Department of Microbiology, University of Minnesota, secretes into the medium an enzyme (or other factors) which lyses heat-killed or freeze-thawed myxospores.

Labelling methods. Vegetative organisms were labelled by adding [32 P]orthophosphate (60 Ci/mg P; 1 Ci/25 ml culture). After 5 min, chloramphenicol (1 mg/ml) and sodium azide (to 1 mM) were added and the cells were poured on to crushed ice and harvested. Myxospore formation was induced in 25 ml cultures by adding glycerol (Bacon & Rosenberg, 1967) and after 4.5 h, [32 P]orthophosphate was added as above. After 15 min phosphate buffer was added to 10 mM. In some experiments the bacteria were poured onto crushed ice and harvested and washed with phosphate buffer as above; in others, the cultures were returned to the orbital shaker and harvested after a further 2 h. We confirmed that the cysts were mature, resistant, refractile spheres by observing them under the phase-contrast microscope and establishing that they were unchanged in appearance and were also viable after sonication. They are referred to as 'pulse-chased myxospores'. They were normally harvested as described by Foster & Parish (1973); for certain experiments they were harvested and washed aseptically and returned to fresh casitone medium containing 10 mM-potassium phosphate buffer; these organisms were then harvested after 15 min at 30 °C.

Isolation of RNA. RNA was isolated from vegetative organisms by a modification (Parish, 1968) of the method of Kirby, Fox-Carter & Guest (1967). The preparations contained all the RNA species present in the organisms and some of the DNA. RNA was isolated from myxospores by several methods which are summarized below.

Freeze-thawed methods. The myxospores were freeze-thawed by immersion successively

in solid CO₂/ethanol and water (30 °C) 10 times. In the 'urea method' the freeze-thawed cells were treated with 8 M-urea, 10 % (v/v) 2-mercaptoethanol (Sakakibara, Tanooka & Terano, 1970), then extracted by the method of Kirby *et al.* (1967). In the 'freeze-thawed/phenol method' the freeze-thawing was performed in the presence of the deproteinizing mixture used by Kirby *et al.* (1967).

Bacillus sp. no. 4 enzymes. The following procedure is that of D. S. Weisberg & M. Dwor-kin (personal communication). The *Bacillus* strain was grown in 1 % (w/v) Bacto-casitone, 10 mM-potassium phosphate buffer, pH 7.4, to an absorbance of 2.0 at 540 nm. The bacteria were removed by centrifugation and the supernatant dialysed against solid polyethylene glycol 6000 at 4 °C to reduce the volume to 0.2 vol. It was then dialysed against 0.1 M-potassium phosphate buffer, pH 7.5 for a total of 21 h with one change of buffer. Two volumes of cold acetone (−10 °C) were added slowly. The precipitate was removed by centrifugation, dissolved in 10 mM-tris HCl pH 7.4 (20 ml) and dialysed against the same buffer. For lysis of myxospores (or assay of the preparation) the following components were incubated for 20 min at 37 °C: myxospores in 1 mg/ml chloramphenicol solution (freeze-thawed four times; 1.60 ml), 0.1 M-potassium phosphate buffer, pH 7.5 (0.04 ml), 1 mg/ml chloramphenicol (0.64 ml), water, (0.77 ml), 1 M-MgCl₂ (0.20 ml), *Bacillus* sp. no. 4 preparation (0.05 ml), 10 % (w/v) sodium cholate (0.30 ml), 0.4 mg/ml polyvinyl sulphate (0.2 ml). The drop in $E_{540\text{nm}}$ was measured. For isolation of RNA, the suspension was extracted by the method of Kirby *et al.* (1967). Ribonuclease was assayed by 'Method 1' of Uchida & Egami (1967). In one experiment, the preparation of *Bacillus* 'enzyme' was fractionated by column chromatography. A sample containing 1 mg protein was applied to a column (10 cm × 1 cm) of DEAE-cellulose, equilibrated in 0.01 M-tris HCl, pH 7.4, and eluted with a linear gradient of 0 to 1 M-NaCl (in the same buffer). The absorbance at 280 nm of the eluate was monitored and fractions were assayed for ribonuclease and myxospore lytic activity.

Silica and alumina grinding methods. The deproteinization procedure is essentially that of Parish & Kirby (1966). 'Phenol mixture' is made by liquefying phenol (500 g) by addition of *m*-cresol (70 ml), water (55 ml) and 8-hydroxyquinoline (0.5 g). Washed vegetative organisms or myxospores were suspended in 6 % (w/v) sodium 4-aminosalicylate, 1 % (w/v) NaCl, 1 % (w/v) sodium triisopropyl-naphthalene sulphonate, 2 mM-ethylenediamine tetracetic acid disodium salt, 3 % (v/v) phenol mixture ('solution A'; 2 ml). The suspension was transferred to a mortar and over a period of 15 min at 0 °C silica or alumina was added in small amounts and ground with a pestle until the material formed a stiff paste. The paste was extracted with 10 ml solution A and 10 ml phenol mixture at room temperature for 20 min, the phases were separated by centrifugation at 4 °C and the upper phase removed with a Pasteur pipette. Solid NaCl (2 %, w/v) was dissolved in this phase which was then extracted with 0.5 vol. phenol mixture for 10 min at room temperature. The phases were separated as before and the upper phase added to 2 vol. ethanol at −20 °C. The precipitate (nucleic acid) was removed by centrifugation, washed twice with 2 % (w/v) NaOAc.3H₂O in 75 % (v/v) aqueous ethanol and dissolved in electrophoresis buffer at 4 °C. Any insoluble material (mucopolysaccharide) was removed by centrifugation.

Fractionation of RNA. RNA was fractionated by polyacrylamide-gel electrophoresis according to the procedures of Loening (1968) with the minor modification described by Parish, Khairul Bashar, Brown & Brown (1971). Details of running conditions are described in the legends to Figures and Loening (1968). Gels were stained and destained (Parish *et al.* 1971) and scanned in the Joyce-Loebl Chromoscan using the 5–045 orange filter. The gels were frozen, sliced into 1 mm slices and counted in water (Cherenkov radiation) in a Beckman liquid scintillation spectrophotometer to a standard error of 2 %.

Fig. 2. Fractionation of labelled RNA by electrophoresis on 3.3 % polyacrylamide gels. Details of labelling methods and preparation and fractionation of RNA are described in Methods. Continuous lines are stained nucleic acid; histograms ^{32}P -radioactivity.

(a) Pulse-labelled vegetative organisms; (b) pulse-labelled 4 to 5 h RNA in organisms undergoing myxospore induction; (c) pulse-chased RNA from mature myxospores; (d) RNA from myxospores (as in c) germinated for 15 min.

Table 1. *Evaluation of methods for isolating RNA from Myxococcus xanthus myxospores*

Preparation	Ratio 23S RNA/ 16S RNA	Zones characteristic of rRNA degradation products	Proportion of RNA in 4S/5S region
Vegetative organism control	2:1	None	20 %
Freeze-thawed/urea	None	None	Present
Freeze-thawed/phenol	None	Several	40 to 70 %
Freeze-thawed/ no. 4	1:1	Many	Approx. 50 %
Alumina/phenol	2:1	Some	Variable
Silica/phenol	2:1	None	20 %

Fig. 3. Fractionation of labelled RNA by electrophoresis on 2.6% polyacrylamide gels. Continuous lines are stained nucleic acid. Zone A is an artefact (not blue stain) presumed to be due to mucopolysaccharide. Histograms are ^{32}P -radioactivity.

(a) Pulse-labelled vegetative organisms; (b) pulse-labelled 4 to 5 h RNA in organisms undergoing myxospore induction; (c) pulse-chased RNA from mature myxospores; (d) RNA from myxospores (as in c) germinated for 15 min.

RESULTS

Isolation of RNA from myxospores. Unlabelled RNA isolated from myxospores by a variety of procedures and analysed by electrophoresis in 3.3% polyacrylamide gels was compared with the total RNA isolated from vegetative cells (Table 1). In the methods using freeze-thawed bacteria, low-molecular weight RNA is preferentially extracted; *Bacillus* sp. no 4 'enzyme' is potentially useful for lysing myxospores and releasing high-molecular weight RNA, but we were unable to prevent some degradation of the RNA. This degradation may occur during the freeze-thawing. Preparations of this 'enzyme' contain 100 to 200 units/ml of ribonuclease and it is unlikely that any ribonuclease inhibitor will totally protect RNA. Fractionation of the *Bacillus* sp. no. 4 preparation by ion-exchange chromatography of DEAE-cellulose yielded several peaks of protein. Ribonuclease is eluted between 0.1 and 0.15 M-NaCl. We were unable to detect any myxospore lytic activity in fractions

of eluate. The observed lytic activity may be due to a combination of two or more enzyme activities.

Attempts at grinding the myxospores with alumina in the presence of the deproteinizing mixture gave variable yields of nucleic acid. Poor recoveries of RNA were obtained when very small amounts of material (as are used for experiments on radioactively labelled myxospores) were employed, perhaps due to binding of nucleic acids to basic sites in the alumina. Acid-washed silica powder was examined as a possible alternative grinding material. We have consistently obtained reproducible yields of undegraded RNA using this technique.

Fractionation of labelled RNA. The results of fractionating RNA on 3.3% and 2.6% gels are summarized respectively in Fig. 2 and 3.

Heterodisperse RNA (possibly mRNA) migrating between the 16 S and 5 S zones is present in pulse-labelled RNA from vegetative organisms and those undergoing morphogenesis (Fig. 2 *a*, 2 *b*, 3 *a*, 3 *b*). This material has largely been eliminated in the mature myxospores (Fig. 2 *c*, 3 *c*). Transfer RNA and 5 S rRNA synthesized during the 4 to 5 h period (Fig. 2 *b*) are present in the mature myxospores (Fig. 2 *c*).

In both types of pulse-labelled organisms 23 S rRNA is labelled. There is radioactivity in zones (16-P and 23-P) characteristic of rRNA precursors (Fig. 3 *a*, *b*; Hecht & Woese, 1968). In the mature myxospores, no 23-P RNA is present but only a proportion of the 16-P RNA has been converted to 16 S rRNA (Fig. 3 *c*). This proportion is increased during the first 15 min of germination; there are no other substantial changes in the labelled RNA during this period (Figs. 2 *d* and 3 *d*). We have neglected any changes in the zones X and Y (Fig. 3) as these are probably aggregates of rRNA (Parish, 1972).

DISCUSSION

The burst of RNA synthesis late in myxospore induction is possibly analogous to the RNA that is synthesized late in the division cycle during vegetative growth. *Myxococcus xanthus* has a DNA synthetic phase corresponding to 0.8 of the growth cycle (Zusman & Rosenberg, 1970); there is synthesis of both 'stable' (rRNA and tRNA) and 'unstable' RNA synthesis in the period between the end of the DNA synthetic phase and the physical separation of the organisms (Zusman & Rosenberg, 1971), even though the rate of protein synthesis increases linearly throughout the cycle (Zusman, Gottlieb & Rosenberg, 1971).

Our data imply that 4 to 5 h RNA contains all classes of RNA but that the 'stable' RNA in the mature myxospores is tRNA (transfer RNA) rRNA and rRNA precursors. The turnover of rRNA correlates with evidence that at least the 30 S ribosomal subunit is different in the myxospores (Foster & Parish, 1973). We believe that demonstration of high-molecular weight RNA synthesis in myxospores requires disruption of the cells (by silica grinding) in the presence of potent nuclease inhibitors (phenol and triisopropyl-naphthalene sulphonate) and accounts for the failure of Ramsay & Dworkin (1970) to observe this material. In experiments (not reported in detail here) in which labelled RNA was extracted by the 'Bacillus sp. no. 4 method', we were unable to obtain any ribosomal precursor RNA. We have no evidence that heterodisperse RNA (characteristic of mRNA) synthesized during the 4 to 5 h period is present in the mature myxospores. The question of whether stable mRNA is present in myxospores remains open. As actinomycin D failed to inhibit germination, Ramsay & Dworkin (1968) concluded that stable mRNA was present in the myxospores. This conclusion may need to be re-evaluated in the light of the selective inhibition of certain, but not all, classes of RNA by the concentration of the antibiotic involved (M. Dworkin, personal communication). The type of transcriptional control effected by a modification to the

σ -factor postulated in sporulation in *Bacillus subtilis* (Losick & Sonenshein, 1969) is probably not involved during myxospore induction (Okano *et al.* 1972). Our studies (Foster & Parish, 1973) imply that translational control operates in myxospores. The present paper suggests that RNA synthesis required for the assembly of myxospore ribosomes occurs during the 4 to 5 h period.

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