Ribosomes, Ribosomal Subunits and Ribosomal Proteins from Myxococcus xanthus

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SUMMARY

Polyribosomes and ribosomes were isolated from vegetative Myxococcus xanthus FB and myxospores. The sedimentation coefficients of monoribosomes and ribosomal subunits were the same as those for equivalent particles from Escherichia coli. Conditions for the dissociation of the ribosomes and the unfolding of the subunits (in strong salt or very low concentrations of magnesium salts) differed from those required for analogous processes in E. coli. The stability of the 30 S subunits differed in preparations from vegetative M. xanthus and myxospores. Spermidine promoted the unfolding of the larger (50 S) subunit in low-magnesium buffer.

Proteins were isolated from ribosomal subunits of Myxococcus xanthus, fractionated by polyacrylamide-gel electrophoresis and a catalogue was compiled. The proteins derived from 'native' subunits and from subunits partly unfolded in strong salt and from ribosomal subunits derived from vegetative cells and myxospores were compared. A difference in the profile of proteins from the 30 S subunits of myxospores and vegetative cells was found. The significance of these results is discussed in the light of the dissociation properties of the ribosomes and also the morphogenetic cycle of M. xanthus.

INTRODUCTION

The conversion of vegetative organisms to myxospores (the resting form of Myxococcus xanthus, referred to as microcysts in some literature) is part of the fruiting cycle of the lower myxobacteria. The process can be synchronously induced in Myxococcus in liquid culture by adding alcohols to the medium (Sadler & Dworkin, 1966). Dworkin & Gibson (1964) pointed out the unique advantages of this process (and the converse process of germination and outgrowth of myxospores) for studying microbial differentiation.

We have studied the possible role of ribosome structure in the differentiation process. In this paper we report the properties of ribosomes and ribosomal proteins from Myxococcus xanthus and the synthesis of ribosomal RNA in relation to the morphogenetic cycle is reported in the following paper. In addition to establishing techniques required for isolating ribosomes and their subunits, we have studied the effect of polyamines on the ribosomes. Polyamines bind to bacterial ribosomes (Stevens, 1970) and in the case of M. xanthus, there is a special interest in the possible effects of polyamines on ribosome properties as putrescine promotes myxospore formation and spermidine inhibits it (Witkin & Rosenberg, 1970).
METHODS

Materials. Bacteriological media were obtained from Difco Laboratories, Detroit, Michigan, U.S.A., Tris base from Sigma Chemical Co., London, chloramphenicol from Parke-Davis Ltd, Hounslow, Middlesex, and radiochemicals from The Radiochemical Centre, Amersham, Buckinghamshire. Silicone MS Antifoam A was obtained from Hopkin & Williams Ltd, Chadwell Heath, Essex, and bacteriological grade alumina A-305 was a gift from Alcoa International S.A., Lausanne, Switzerland. AnalaR urea was purified by passing a solution (approx. 9 M) through a column of Amberlite Monobed resin MB-3 and stored at −20 °C; it was thawed and diluted when required for dialysis of ribosomal proteins. Solid urea (for use in polyacrylamide gel electrophoresis) was obtained by crystallizing the purified 9 M solution at 0 °C.

Dyes and stains were obtained from George T. Gurr Ltd, London.

Bacteria. Myxococcus xanthus FB (NCIB 9412 and ATCC 19368) was a gift from Professor M. Dworkin, Department of Microbiology, University of Minnesota. It was maintained in liquid culture and periodically fruiting and germinated on solid media by the method of Dworkin (1962). The organism was grown in 1 % (w/v) Bacto-casitone, 0·2 % MgSO₄·7H₂O. For experiments in which RNA was to be labelled with [³²P]orthophosphate, the cells were grown in 1 % (w/v) deionized casitone supplemented with MgSO₄ and salts of the medium of Garen & Levinthal (1960). Deionized casitone was made by passing a solution (approx. 20 %, w/v) of Bacto-casitone through a column of AG 11A8 ion-retardation resin. Unbound phosphate in the eluate was assayed by the procedure of Fiske & Subba Row as described by Leloir & Cardini (1957) and fractions containing peptides, eluted before the bulk of the inorganic phosphate, were pooled and freeze-dried. A solution (1 %, w/v) of this product contained less than 10⁻⁵ M-inorganic phosphate.

For large cultures, it is essential to prevent both foaming of the medium and clumping of the bacteria. We have routinely used 4 l cultures agitated with a large Teflon-covered magnetic stirrer and vigorously aerated. Foaming was controlled by suspending a glass ‘spoon’ containing antifoam approx. 10 cm above the surface of the culture fluid. The bacteria were grown (from an inoculum of 100 ml) at 28 °C and harvested in mid- or late-exponential phase (E₅₆₀ nm between 1·0 and 2·0) by pouring the culture on to crushed ice and centrifugation at 0 °C.

Myxospores were produced by adding glycerol to 0·5 M to exponentially growing bacteria in the casitone medium described above (Bacon & Rosenberg, 1967). For this purpose, the bacteria were grown in 1·5 l batches in a giant testtube, 75 cm × 7 cm (internal dimensions). The culture was vigorously aerated with a wide-pore sinter at the bottom of the tube. Foaming was controlled in the way described for growth of the vegetative organism. When the culture had reached mid-exponential phase (E₅₆₀ nm of 1·0) the requisite amount of warmed glycerol, mixed with enough water to make the liquid easy to pour, was added aseptically. The organisms were rounded at 2 h, phase-refractile at 4 h and were harvested at 6 to 8 h. Approximately 90 to 95 % were mature myxospores. They were washed by centrifugation successively in 1 M-KCl and distilled water. These conditions lyse the remaining vegetative bacteria.

Escherichia coli MRE 600 was grown in the medium of Garen & Levinthal (1960). Sodium β-glycerophosphate forms the only phosphorus source; the organism was labelled with carrier-free [³²P]orthophosphate (50 Ci/mg P; 5 μCi/ml) for 2 h. It was harvested by centrifugation and pooled with vegetative Myxococcus xanthus or myxospores and ribosomes isolated as described below.
Myxococcus ribosomes

Isolation of polyribosomes. Polyribosomes were isolated from vegetative cells by a procedure devised for Escherichia coli ’Method II’ of Parish (1969) with two modifications. The bacteria were harvested (in the presence of chloramphenicol and sodium azide) but were not treated with lysozyme; they were suspended and lysed directly with 1% (v/v) Triton X-100 (in place of deoxycholate). Thereafter the polysomes were isolated as pellets, following centrifugation through layers of sucrose as described by Parish (1969).

Isolation of ribosomes. Ribosomes were isolated from both myxospores and vegetative bacteria by the same procedure. All operations were performed at 0 °C except for the centrifugations which were performed at 4 °C. The bacteria were washed in 50 mM-KCl, 10 mM-Mg(OAc)₂, 10 mM-tris-HCl, pH 7-4, 6 mM-2-mercaptoethanol (TKM) and ground with 2.5 x their wet wt of alumina. The grinding was performed by putting the bacteria into a mortar and adding alumina a little at a time whilst grinding with the pestle to achieve an even paste. The grinding was continued for 5 min; TKM buffer (1.5 x the wet wt of bacteria) was added slowly with continued grinding to form an even slurry which was left for 15 min and then centrifuged at 15,000 g for 15 min. The supernatant was removed and centrifuged at 30,000 g for 30 min in the 30 rotor of the Beckman model L2-65B ultracentrifuge. The upper two-thirds of the supernatant was removed with a Pasteur pipette (S 30 fraction). The ribosomal pellets were obtained by centrifuging the S 30 fraction at 145,000 g in the Ti50 rotor (for the same centrifuge) for 2 h.

For certain preparations these ribosomal pellets, ‘unwashed ribosomes’, were used directly for fractionation. For other experiments the ribosomal pellets were dispersed in 0.5 M-NH₄Cl, 10 mM-Mg(OAc)₂, 60 mM-KCl, 10 mM-tris HCl, pH 7.4, 6 mM-2-mercaptoethanol and centrifuged at 4 °C at 165,000 g for 2.5 h in the Ti50 rotor of the Beckman model L2-65B ultracentrifuge. The supernatant was removed and the pellets washed again by using the same procedure. The final pellets were used for fractionation. These preparations are referred to as ‘NH₄Cl ribosomes’.

Fractionation of ribosomes and polyribosomes. Details of buffers used and conditions for sucrose-gradient centrifugation appear in the legends to the appropriate figures. The gradients were monitored by pumping them through a flow-through cell of the Gilford 2000/Unicam SP 500 automatic recording spectrophotometer. Radioactivity was measured by diluting the fractions with water (5 ml) and counting the Cherenkov radiation in a Beckman liquid scintillation spectrophotometer to a standard error of 7%.

In the case of the polyribosome gradients, an equal volume of trichloroacetic acid (10%, w/v) was added to each fraction, the precipitates were collected by filtration, washed with 5% (w/v) trichloroacetic acid and were suspended in water for Cherenkov counting.

Ribosomal subunits were fractionated on a preparative scale in a zonal rotor. Details are given in the legend to Fig. 4. The gradients were pumped out of the rotor by displacement with 50% (w/v) sucrose pumped to the wall of the rotor and fractions (15 ml) were collected on ice. Samples were diluted and their absorbance at 280 nm measured. Subunits were recovered from selected fractions by dialysis versus TKM buffer containing 10% (w/v) polyethylene glycol 6000 overnight at 4 °C. The non-diffusible material was centrifuged at 165,000 g and 4 °C for 5 h. The ribosomal subunits were collected as pellets.

Isolation and fractionation of ribosomal protein. Proteins were obtained from pellets of ribosomal subunits by a method based on that of Waller (1964). The ribosomes (or subunits) were resuspended in 10 mM-MgCl₂, 5 mM-tris HCl, pH 7.4, to give 20 mg ribosomes/ml. Two volumes of glacial acetic acid were added at 0 °C over a period of 15 min with continuous agitation. After standing at 0 °C for 2 to 3 h (with occasional stirring), the mixture was centrifuged for 15 min at 10,000 g and 4 °C to remove the precipitated RNA. The pellets...
Fig. 1. Polyribosomes from vegetative cells of *Mycobacterium xanthus*. Polyribosomes were isolated from cells labelled for 5 min with [32P]orthophosphate by the procedures described in Methods. The pellets were suspended in TKM buffer and layered over linear gradients of sucrose (15 to 60 %, w/v) in TKM established in 35 ml tubes of the SW27 rotor for the Beckman L2-65B ultracentrifuge. Centrifugation was for 2 h at 27000 rev./min and 4 °C.

Panel (a) shows a control experiment; in (b) the same suspension of polyribosomes was incubated at 30 °C with ribonuclease (5 µg/ml) for 10 min prior to layering over the gradient. Absorbance is represented as a continuous line and radioactivity as a histogram.

were re-extracted with a small volume of 66 % (v/v) acetic acid. The acetic-acid supernatants were dialysed versus 8 m-urea for 15 h at 4 °C. The non-diffusible solutions were adjusted to pH 5·0 (by addition of 1 M-KOH), dialysed again versus fresh 8 m-urea, and stored at 4 °C.

Ribosomal proteins were fractionated by polyacrylamide disc electrophoresis using the method of Leboy, Cox & Flaks (1964). The gels (10 cm × 0·6 cm) were normally run at 2·5 mA/gel at room temperature until a marker (pyronin Y) had migrated to the end of the gel (approx. 4 h); alternatively electrophoresis was extended for a further 2 to 3 h. The gels were stained by immersion in a solution of 0·5 % (w/v) amido black in ethanol: acetic acid:water (5:1:7, by vol.) for 1 to 2 h at room temperature, and destained (24 to 48 h) in the same solvent in a device in which solvent was pumped over the gels, passed through a
M. xanthus ribosomes

Fig. 2. A comparison of ribosomes from Myxococcus xanthus with those from Escherichia coli. Ribosomes were suspended in 50 mM-KCl, 10 mM-tris HCl, pH 7.4, 6 mM-2-mercaptoethanol containing Mg(OAc)$_2$ either 10 mM (a, b) or 0.25 mM (c). They were centrifuged through linear gradients of sucrose (15 to 35 % w/v), in the buffer used for suspending the ribosomes, in 35 ml tubes of the SW27 rotor for the 15.5 h at 20500 rev./min and 4 °C. In each figure, absorbance (continuous line) is due to M. xanthus ribosomes and radioactivity (histogram) to E. coli ribosomes (see Methods).

(a) Vegetative-cell ribosomes in 10 mM-Mg(OAc)$_2$; (b) myxospore ribosomes in 10 mM (MgOAc)$_2$; (c) vegetative-cell ribosomes in 0.25 mM-Mg(OAc)$_2$.

short column of Dowex-1 X 50 and re-circulated. The gels were scanned in a Joyce–Loebl Chromoscan using a red filter (620 nm).

RESULTS

Properties of polyribosomes from vegetative cells. Analysis of polyribosomes labelled with $^{[32P]}$orthophosphate by sucrose-gradient centrifugation (Fig. 1) showed that the pellet contains, in addition to polyribosomes, monoribosomes and small amounts of ribosomal subunits. Treatment with a small amount of ribonuclease converted the polyribosomes to ribosomes (Fig. 1 b). As very little radioactivity was found associated with the 70 S monoribosome peak, following treatment with ribonuclease, we conclude that after 5 min, the bulk of the label is incorporated into mRNA (messenger RNA) and not into tRNA (transfer RNA) nor rRNA (ribosomal RNA).

Sedimentation properties of ribosomes and ribosomal subunits. The dissociation properties of unwashed ribosomes from vegetative Myxococcus xanthus and myxospores were compared with those for Escherichia coli ribosomes by using ribosomes obtained from mixtures of M. xanthus cells and trace amounts of $^{32P}$-labelled E. coli (Fig. 2). The sedimentation coefficients of monoribosomes (70 S) and subunits (30 S and 50 S) are the same as those for the E. coli particles. In low-magnesium buffer the ribosomes from both E. coli and vegetative M. xanthus are totally dissociated into subunits (Fig. 2 c). An identical result was obtained with ribosomes from myxospores. There is a striking difference between the profiles obtained from M. xanthus (either form) and E. coli in high-magnesium buffer. Very few M. xanthus monoribosomes are seen in the gradient.

Lowering the magnesium-ion concentration to 0.1 mM and the presence of sodium salts caused the 30 S subunits from vegetative cells of Myxococcus xanthus to unfold and yield slowly sedimenting particles; the same effect is not found for myxospore subunits (Fig. 3 a,
Fig. 3. Effects of very low magnesium-salt concentration, NaCl and spermidine on *Myxococcus xanthus* ribosomal subunits. In all cases the same buffer was employed for suspending ribosome pellets and in the sucrose gradients. Conditions for centrifugation were the same as in Fig. 2.

The panels show ribosomes from vegetative cells (a) and myxospores (b) in 60 mM-NH₄Cl, 10 mM-tris HCl, pH 7.4, 0.1 mM-Mg(OAc)₂, 6 mM-2-mercaptoethanol; and from vegetative cells (c) and myxospores (d) in 60 mM-NH₄Cl, 10 mM-tris HCl, pH 7.4, 0.4 M-NaCl, 10 mM-Mg(OAc)₂, 6 mM-2-mercaptoethanol. Panel (e) shows ribosomes from vegetative cells in 60 mM-NH₄Cl, 10 mM-tris HCl, pH 7.4, 0.1 mM-Mg(OAc)₂, 6 mM-2-mercaptoethanol, 4 mM-spermidine hydrochloride.

Similarly 0.4 M-NaCl unfolds the vegetative cell 30 S subunits but not those from myxospores (Fig. 3c, d). In the presence of spermidine (4 mM), exposure to very low concentrations of magnesium ions results in both 30 S and 50 S subunits being converted to low sedimenting forms (Fig. 3e). Spermidine has no effect on the sedimentation properties of the subunits in 10 mM-magnesium ions.

Typical fractionations in the zonal ultracentrifuge of unwashed and NH₄Cl ribosomes from vegetative bacteria are illustrated in Fig. 4. Identical results were obtained from myxospore preparations. The unfolding of the 30 S subunit is effected by washing the ribosomes with ammonium chloride. This result emphasizes the ease with which the unfolding occurs. Washing of *Escherichia coli* ribosomes under these conditions results in the release of certain proteins (notably ribonuclease) but no unfolding occurs (Voigt & Matthaei, 1968).

**Fractionation of ribosomal proteins.** No differences were found between the proteins from the 50 S subunits of vegetative *Myxococcus xanthus* and myxospores (Fig. 5a, b). These
Fig. 4. Fractionation of ribosomal subunits in the zonal ultracentrifuge. A buffer of 50 mM-KCl, 0.25 mM-Mg(OAc)$_2$, 10 mM-tris HCl, pH 7.4, 6 mM-2-mercaptoethanol was present in all solutions. A gradient of total volume 500 ml, linear with volume, of sucrose (7.5 to 30% w/v) was established in the BXIV Ti rotor of the MSE Superspeed 65 ultracentrifuge. An underlay of 45% (w/v) sucrose was pumped into the rotor. Ribosomes (approx. 0.1 g) suspended in 2% (w/v) sucrose (30 ml) were introduced via the centre of the rotor. An overlay of buffer (80 ml) was pumped on. The centrifuge was accelerated to 45000 rev./min and run at this speed for 4 h at 5°C. (a) Unwashed ribosomes; (b) NH$_4$Cl ribosomes. Fractions were pooled corresponding to the zones shown.

proteins are referred to as the L-proteins (from the large subunit). We have not regarded as significant, differences in the minor (presumably transitory) proteins, L1, L5, L6, L15a, L15b, L15c and L16, as these bands vary in intensity in separate preparations (from the same cell type). The profile of L-proteins from NH$_4$Cl ribosomes was the same except L1 and L6 are removed by washing. Analysis of the proteins by prolonged electrophoresis (details in Methods) shows that it is possible to resolve L9, L10, L11, L12, L13 and L14 into two bands each and L8 into three bands. The maximum number of L-proteins resolved in this system is therefore 26.

The fractionation of the S-proteins (from the 30 S or smaller subunit) resolves 17 bands of which S1 has 'run off' the gels illustrated and the pairs S14/S15 and S16/S17 are unresolved. In analyses of independent preparations we find that in myxospores S6 is consistently higher than S7 whereas the reverse is true of vegetative bacteria; likewise S4 is strong in myxospores and weak in vegetative bacteria (see Fig. 5c and d). The other difference illustrated in the Figure (increased strength of S3 in the myxospores) is possibly insignificant, as the fast migrating minor proteins (S1, S2, S3) are variable (compare L1, L5 and L6 above).

The S-proteins of the two particles (see Fig. 4) derived from 30 S subunits in NH$_4$Cl
Fig. 5. Ribosomal proteins of *Myxococcus xanthus* separated by gel electrophoresis (see Methods for experimental details). All the labelled zones were discernible by eye although some are not resolved by the gel scanner.

Sources of proteins were (a) 50 S subunits from vegetative bacteria, (b) 50 S subunits from myxospores, (c) 30 S subunits from vegetative bacteria and (d) 30 S subunits from myxospores. All these (a–b) were obtained from unwashed ribosomes (see Fig. 1a). The remaining panels show proteins from regions I(e) and II(f) from NH₄Cl preparations (see Fig. 4b).

Ribosomes are compared (Fig. 5e and f); both particles have lost some protein (notably S1, S2, S3 and some of S6 and S7) and S5 is completely removed from the very light (unfolded) particle. Profiles of proteins from these particles show intense, slowly migrating zones. These are probably due to aggregates but we have been unable to dissociate them with detergents.

**DISCUSSION**

The ribosomes of *Myxococcus xanthus* differ from those of *Escherichia coli* in several respects. Very few monoribosomes are obtained when preparations from alumina-ground bacteria are centrifuged in TKM buffer. Vegetative *M. xanthus* lysed with detergent (‘polyribosome preparations’) shows a substantial amount of monoribosomes. We conclude that a very active ribosome dissociation factor is released during the alumina grinding.
The 30 S ribosomal subunits of *Myxococcus xanthus* vegetative cells are susceptible to unfolding in NaCl or very low magnesium-ion concentrations. The conditions employed dissociate *Escherichia coli* ribosomes into subunits but stronger conditions are required for unfolding the subunits (Gavrilova, Ivanov & Spirin, 1966; Gesteland, 1966; Miall & Walker, 1969). The implications of these results for an understanding of ribosome structure cannot be evaluated at present. However the greater stability of the 30 S subunits from myxospores and the differences in the S-proteins imply a difference in these subunits in the two cell types. This difference correlates with the observation that germination of myxospores is less sensitive to streptomycin (an inhibitor of protein synthesis which binds to the 30 S subunit) than is vegetative growth (Ramsay & Dworkin, 1968).

The effect of spermidine on ribosomal subunits from *Myxococcus xanthus* should be taken into account when evaluating the significance of its inhibitory effect on myxospore induction. Witkin & Rosenberg (1970) supposed this to be related to the effect of methionine-starvation on morphogenesis, implying that control of C1 metabolism (via the S-adenosyl methionine pool) was involved in the process. An alternative explanation of the effects of polyamines is that if vegetative-form ribosomes are turned over in response to damage by spermidine, the production of ‘myxospore ribosomes’ may be inhibited.

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