16s and 23s Components in the Ribosomal Ribonucleic Acid of *Rhodopseudomonas spheroides*

By J. F. SZILÁGYI

Chester Beatty Research Institute, Institute of Cancer Research:
Royal Cancer Hospital, London, S.W. 3

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1. Ribosomal RNA was extracted from lysates of *Rhodopseudomonas spheroides* without prior isolation of ribosomes. 2. The composition of this RNA was investigated by using gradient centrifugation, showing that the proportion present as 23s component depended on the method of extraction. 3. The highest proportion of 23s component was found when cells were disrupted by ultrasonic treatment in the presence of ribonuclease inhibitors. 4. The results indicated that a ribonuclease is active in the cell lysate; this could account for the previous report (Lessie, 1965) that ribosomes of *Rhodopseudomonas spheroides* do not contain a 23s component.

The photosynthetic bacterium *Rhodopseudomonas spheroides* contains 70s-type ribosomes that can be separated into 50s- and 30s-type sub-units (Lessie, 1965; Friedman, Pollara & Gray, 1966). In this respect *R. spheroides* is similar to other bacteria (Taylor & Storck, 1964).

However, when the ribosomal RNA composition of *R. spheroides* was examined, it was claimed by Lessie (1965) that it contained only the 16s component. The suggestion was then made (Lessie, 1965) that the 23s component might be a dimer of the 16s ribosomal RNA.

The possibility was not ruled out that some degradation of 23s RNA had occurred during the extraction procedure used in the previous work. It was therefore thought desirable to re-examine whether the 23s RNA component was in fact absent from this organism, and in the present investigation RNA was extracted from whole cells rather than from ribosomes.

It could be anticipated that, if a 23s RNA component were found, the use of whole-cell extraction would not establish that this component originated from the ribosomes. The composition of RNA in other cellular fractions is not known with any precision, though Bull & Lascelles (1963) reported that the chromatophore fraction, which originated from the cellular membrane, constitutes only 10% of the total RNA.

It seems reasonable therefore to assume that any fraction of high-molecular-weight RNA that could be isolated in appreciable amount had in fact originated from the ribosomes, though the presence of such RNA in the membrane could not be excluded.

MATERIALS

R. spheroides was obtained from the National Collection of Industrial Bacteria, strain 8253.

Sodium tri-isopropyl-naphthalenesulphonate (technical) was obtained from Kodak Ltd., Kirkby, Liverpool, and lysozyme from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

[2-¹⁴C]Uridine (56.4 mc/m-mole) in aqueous solution was obtained from The Radiochemical Centre, Amersham, Bucks.

Phenol reagent contained phenol (500g.), *m*-cresol (70ml.), 8-hydroxyquinoline (0.5g.) and water (55ml.) (Kirby, Fox-Carter & Guest, 1967).

Ribosomal RNA from *Escherichia coli* strain B was prepared by the method of Kirby *et al.* (1967).

METHODS

Growth of cultures. *R. spheroides* was grown aerobically or photosynthetically in the malate-glutamate medium of Lascelles (1959) containing 0.1% (w/v) of Difco yeast extract in flat medicine bottles or in Roux bottles. Aerobic cultures were grown at 34° with continuous aeration from a small air pump. Photosynthetic cultures were incubated at 34° with continuous gassing with air+N₂ (10:90) mixture, and were illuminated through the glass-sided water bath with two 150w light-bulbs placed 30cm. away from the bottles. The mean generation time was approx. 2hr. during exponential growth for both aerobic and photosynthetic cultures. Concentration of cells was estimated from the relationship, established in separate experiments, that $E_{0.5cm.} = 1.0$ at 680m μ for a suspension containing 1mg. dry wt. of cells/ml.

Labelling of RNA. When the growth of the cultures reached approx. 0.3mg. dry wt./ml. a solution containing [2-¹⁴C]uridine, unlabelled thymidine and cytidine was

added to give a final concentration of $0.03 \mu\text{C}$ of uridine, $7 \mu\text{g}$. of thymidine and $7 \mu\text{g}$. of cytidine/ml. of culture.

Harvesting of cells. The exponentially growing cultures were harvested by centrifugation (3000g for 10 min. at 2°) when they contained about $0.8\text{--}0.9 \text{ mg}$. dry wt./ml.

Extraction of nucleic acids. The method of Kirby *et al.* (1967) was used with minor modifications. The harvested cells were resuspended in a mixed solvent containing sodium tri-isopropyl-naphthalenesulphonate (1%, w/v), NaCl (3%, w/v), sodium 4-aminosalicylate (6%, w/v) and phenol reagent (3%, v/v) and contained approx. 7 mg . dry wt. of cells/ml. After shaking for 5 min. an equal volume of phenol reagent was added and the mixture was further shaken mechanically for 20 min. The phases were then separated by centrifugation (17000g for 10 min.). The upper aqueous layer was removed carefully and sodium tri-isopropyl-naphthalenesulphonate was added to give a final concentration of 1% (w/v). The solution was then shaken for 10 min. with phenol reagent (0.5 vol.). After centrifugation (17000g for 10 min.), the aqueous phase was again carefully removed and then constituted the 'nucleic acid extract'. The procedure was conducted at 2° throughout.

Disruption of cells with lysozyme. Harvested cells were washed once with 0.05 M -tris-HCl buffer, pH 8.5. Cell suspensions containing 7.5 mg . dry wt. of cells/ml. were incubated for 5, 10 or 15 min. at 34° in the presence of 0.1 M -tris-HCl buffer, pH 8.5, containing EDTA (5 mM) and lysozyme (0.5 mg ./ml.) (Lascelles & Szilágyi, 1965).

Disruption of cells. Cells were suspended in 3% (w/v) NaCl solution, precooled to 2° , to give 20 mg . dry wt. of cells/ml. The suspensions (20 ml.) in an ice bath were then treated for 6 min. with a MSE ultrasonic disintegrator at 20 kcyc./sec., 60 w, with the $\frac{3}{8}$ in. diameter vibrator probe; the temperature did not rise above 7° .

Disruption of cells in the presence of ribonuclease inhibitors. Cells were resuspended in a mixture, previously cooled to 2° , containing NaCl (3%, w/v), sodium tri-isopropyl-naphthalenesulphonate (3%, w/v), sodium 4-aminosalicylate (18%, w/v) and phenol reagent (9%, v/v), at a concentration of 20 mg . dry wt./ml. The suspension (20 ml.) was then subjected to ultrasonic disintegration for 6 min., and diluted with cold aqueous NaCl (3%, w/v; 2 vol.). The nucleic acid extraction was then as described above.

Treatment of cells with sodium benzoate and *m*-cresol before extraction of nucleic acids. To exponentially growing cultures, containing about $0.8\text{--}0.9 \text{ mg}$. dry wt. of cells/ml., solid sodium benzoate (40 g ./100 ml. of culture) and *m*-cresol (60 ml./100 ml. of culture) were added and the mixture was shaken for 5 min. at 2° . The suspensions were then centrifuged (17000g for 10 min. at 2°) and the pellets were subjected to ultrasonic disintegration in the presence of ribonuclease inhibitors, and nucleic acid was then extracted as described above.

Purification of ribosomal RNA. To the nucleic acid extract, ethanol (2 vol.) was added, the mixture was kept at -20° for 16 hr. and the resultant precipitate was collected by centrifugation (700g for 10 min.). It was then washed twice with ethanol-water-sodium acetate (75:25:2, v/v/w), twice with 3 M -sodium acetate buffer, pH 6.0, and twice again with the ethanol-water-sodium acetate solution. Ribosomal and rapidly labelled RNA then remained insoluble, whereas the DNA was dissolved (Kirby *et al.* 1967). The RNA was dissolved in 0.15 M -sodium acetate buffer, pH 6.0, and made 4 M with respect to NaCl. The mixture was kept overnight

at -20° and then centrifuged (17000g for 15 min. at 2°), and the precipitated material was washed twice with ethanol-water-sodium acetate (75:25:2, v/v/w) and dissolved in 0.1 M -sodium acetate.

In some cases DNA was precipitated from the nucleic acid extract by very careful addition of *m*-cresol (0.1–0.12 vol.) (Kirby *et al.* 1967) and removed by centrifugation (700g for 10 min.). To the supernatant, 2 vol. of ethanol was added and the solution was stored at -20° for 16 hr., during which time the ribosomal and rapidly labelled RNA precipitated. The resulting gel was further purified as described above.

All steps of the purification were done at 2° unless stated otherwise.

Gradient centrifugation of RNA. Density gradients containing chloral hydrate (10–40%, w/v, in 0.15 M -sodium acetate, pH 7.5) were prepared at 5° in 23 ml. tubes for the SW30 rotor of the MSE TC50 centrifuge, and the RNA solution (1 ml.) was layered on to the top of the gradient. They were centrifuged at 100000g for 13–16 hr. The tubes were then pierced with a MSE needle and 34–36 fractions (28 drops) were collected. From each fraction 0.4 ml. was withdrawn into scintillation counting bottles, 10 ml. of phosphor was added and radioactivity was assayed in a Packard Tri-Carb liquid-scintillation spectrometer (efficiency 45%). The concentrations of nucleic acids in the fractions were determined by ultraviolet absorption, assuming $E_{260}^{1\%} = 160$.

RESULTS

The nucleic acids from *R. spheroides*, including both DNA and RNA, prepared by the method of Kirby *et al.* (1967), remained in solution when stored overnight in the presence of 4 M -sodium chloride. When the nucleic acids were precipitated by ethanol the precipitate dissolved completely in 3 M -sodium acetate. When DNA was precipitated by the addition of 0.12 vol. of *m*-cresol to the extracts, the RNA remaining in the supernatant was also soluble at high salt concentrations. These observations showed that undegraded ribosomal RNA was not present in these extracts, since it is known that undegraded ribosomal RNA from bacteria is insoluble both in 3 M -sodium acetate and in 4 M -sodium chloride (Kirby *et al.* 1967).

The absence of ribosomal RNA was confirmed by density-gradient centrifugation of the nucleic acid extracts containing labelled RNA. All the radioactivity appeared in the region of 4 s (fraction 26–34) (Fig. 1) and was therefore indicated to be present in soluble RNA. When cells, grown aerobically or anaerobically, were treated with lysozyme in the presence of EDTA, 5 min. of incubation resulted in substantial weakening of the cell walls. This was manifested by complete lysis on 20-fold dilution of the cell suspension with water. Density-gradient centrifugation of the nucleic acid extracts of preparations lysed by this method showed that, in addition to soluble RNA, as found when extracts by the method of Kirby *et al.* (1967) were used, other

Fig. 1. Density-gradient centrifugation [in 10–40% (w/v) chloral hydrate in 0.15M-sodium acetate at pH 7.5 at 30000 rev./min. for 15 hr. in the MSE TC50 centrifuge (SW30 rotor) at 5°] of [2-¹⁴C]uridine-labelled RNA from photosynthetically grown *R. spheroides*. The culture was washed once with 0.05M-tris-HCl buffer, pH 8.5. Nucleic acids were then released from the cells with sodium tri-isopropyl-naphthalenesulphonate and sodium 4-aminosalicylate and then extracted with phenol reagent as described in the text. The *S* value was estimated by using the results of Kirby *et al.* (1967) for *E. coli*.

Fig. 2. Density-gradient centrifugation (as described in Fig. 1) of [2-¹⁴C]uridine-labelled RNA from photosynthetically grown *R. spheroides*. The culture was washed once with 0.05M-tris-HCl buffer, pH 8.5, and resuspended in this buffer. Nucleic acids were then released from the cell suspension by incubation for 5 min. with lysozyme, followed by treatment with sodium tri-isopropyl-naphthalenesulphonate and sodium 4-aminosalicylate and extraction with phenol reagent as described in the text. The *S* values were estimated by using the results of Kirby *et al.* (1967) for *E. coli*.

peaks appeared in the region of 16s (fractions 14–21) and 23s (fractions 11–13) (Fig. 2). The 23s peak was smaller than the 16s peak and was completely absent when the cell suspension had been incubated in the presence of lysozyme for longer times, e.g. 15 min.

Fig. 3. Sedimentation analysis in parallel chloral hydrate density gradients [in 10–40% (w/v) chloral hydrate in 0.15M-sodium acetate at pH 7.5 at 30000 rev./min. for 14 hr. in the MSE TC50 centrifuge (SW30 rotor) at 5°] of [2-¹⁴C]-uridine-labelled RNA from *R. spheroides* grown aerobically (—) and of unlabelled ribosomal RNA from *E. coli* (----). A cell suspension (20 mg. dry wt./ml.) was prepared from a culture of *R. spheroides* with 3% (w/v) NaCl. The nucleic acids were then released by ultrasonic treatment for 6 min., followed by treatment with tri-isopropyl-naphthalenesulphonate and sodium 4-aminosalicylate and extraction with phenol reagent as described in the text. To the extract 2 vol. of ethanol was added, and the precipitated nucleic acids, after being washed with ethanol-water-sodium acetate (75:25:2, v/v/w), were redissolved in 0.15M-sodium acetate (15 ml.); 1 ml. was then applied to the gradient.

A closely similar result was obtained when RNA from cells disrupted by ultrasonic treatment in the presence of 0.5M-sodium chloride was used. The 16s RNA was observed to sediment slightly behind the corresponding *E. coli* ribosomal RNA (Fig. 3).

However, when the cells were disrupted immediately after resuspension in a mixture of sodium tri-isopropyl-naphthalenesulphonate, sodium 4-aminosalicylate and phenol solution, two well-defined ribosomal RNA peaks were shown to be present by density-gradient centrifugation (Fig. 4). It was further established that, when resuspension of the cells was not immediately followed by ultrasonic disintegration, the 23s peak became smaller, and occasionally was completely absent. The identity of the two peaks with ribosomal RNA was confirmed by further purification following the procedure described in the Methods section; both RNA components were insoluble in 3M-sodium acetate and in 4M-sodium chloride.

Fig. 4. Sedimentation analysis in parallel chloral hydrate density gradients (as described in Fig. 3) of [^{14}C]uridine-labelled RNA from *R. spheroides* grown aerobically (—) and of ribosomal RNA from *E. coli* (-----). A cell suspension (20mg. dry wt./ml.) containing 3% (w/v) NaCl, 3% (w/v) sodium tri-isopropyl-naphthalenesulphonate and 18% (w/v) sodium 4-aminosalicylate was prepared from a culture of *R. spheroides*. The suspension was ultrasonically treated for 6 min. and diluted with 3 vol. of 3% (w/v) NaCl, and the nucleic acids were then extracted with phenol reagent. To the extract 2 vol. of ethanol was added, and the precipitated nucleic acids, after being washed with ethanol-water-sodium acetate (75:25:2, v/v/w), were redissolved in 0.15M-sodium acetate (15ml.); 1 ml. of the solution was then applied to the gradient.

When cells, after treatment with *m*-cresol and sodium benzoate, as described in the Methods section, were resuspended in a mixture of sodium tri-isopropyl-naphthalenesulphonate, sodium 4-aminosalicylate and phenol reagent before ultrasonic disintegration, density-gradient centrifugation again showed the presence of the two ribosomal RNA components (Fig. 5). This pretreatment of cells with *m*-cresol and sodium benzoate was advantageous in that it gave results that were more reproducible.

DISCUSSION

All bacteria so far examined contain a 70s-type ribosomal RNA that, in appropriate Mg^{2+} concentrations, can be separated into 50s- and 30s-type sub-units (Taylor & Storck, 1964). The ribosomes of *R. spheroides* have a sedimentation coefficient of 66s, with 45s and 29s sub-units (Friedman *et al.* 1966). Though the ribosomes of

Fig. 5. Centrifugation pattern in chloral hydrate density gradient (as described in Fig. 3) of [^{14}C]uridine-labelled RNA from aerobically grown *R. spheroides*. The culture was made 40% (w/v) with respect to sodium benzoate and 0.6 vol. of *m*-cresol was added. After ultrasonic treatment for 6 min. and centrifugation, the pellet was treated with sodium tri-isopropyl-naphthalenesulphonate and sodium 4-aminosalicylate and the nucleic acids were extracted with phenol reagent. Nucleic acids were precipitated by addition of 2 vol. of ethanol to this extract, and after being washed with ethanol-water-sodium acetate (75:25:2, v/v/w), were redissolved in 0.15M-sodium acetate (15ml.); 1 ml. was then applied to the gradient.

R. spheroides are thus of the characteristic bacterial type, Lessie (1965) reported that they contained only one of the usual ribosomal RNA components, namely that corresponding to the 16s ribosomal RNA of *E. coli*; the 23s ribosomal RNA was not found. Similar anomalous ribosomal RNA composition was reported in another photosynthetic bacterium, *Rhodospseudomonas capsulata*. However, other photosynthetic bacteria, *Rhodospseudomonas pallustris* and *Rhodospirillum rubrum* contained both 16s and 23s ribosomal RNA components.

In the work of Lessie (1965), partially purified ribosome preparations were made before extraction of RNA. It seemed possible that some degradation of RNA could have occurred during this procedure, and therefore it was decided to extract the nucleic acids, including ribosomal RNA, from whole cells. In this way ribonuclease action would be minimized.

In preliminary experiments in which nucleic acids were extracted from *R. spheroides* by the method described for *E. coli* (Kirby *et al.* 1967), the presence of ribosomal RNA could not be detected. The possibility that the ribosomal RNA, though extracted, had escaped detection by being soluble in concentrated salt solutions was eliminated by

density-gradient centrifugation of the nucleic acid extracts containing labelled RNA. As no ribosomal RNA could be shown by either of these methods, it was clear that, though DNA and soluble RNA were extracted by this method, ribosomal RNA was not.

The cells were therefore treated with lysozyme, resulting in complete lysis. Subsequent extraction then liberated nucleic acids containing the ribosomal component. However, when this was examined by density-gradient centrifugation the findings of Lessie (1965) appeared to be confirmed. Only 16s ribosomal RNA was present, though very small quantities of 23s ribosomal RNA could also be detected when the time of incubation in the presence of lysozyme was kept short. This indicated that 23s RNA might have been present in the cells, but had been degraded. Either the low ionic strength of the cell suspension had caused denaturation of the 23s RNA, thus altering its sedimentation characteristics, or during incubation preferential enzymic breakdown of this RNA had occurred. Presumably, in either case, the sedimentation characteristics of the structurally altered 23s RNA had become similar to those of the 16s RNA. This could account for the wide base of the 16s peak and the fact that it had a slightly lower sedimentation coefficient than that of the 16s ribosomal RNA of *E. coli*.

Another method of extraction was therefore used, in which cells were disrupted by ultrasonic treatment in the presence of the extraction mixture. The isolated RNA then definitely contained the 23s component. As the extraction was made in the presence of 0.5M-sodium chloride, configurational changes must have been minimal. The amount of the 23s component remained, however, smaller than that of 16s component. This result contrasts with the finding that in *E. coli* the 23s component predominates (Kirby *et al.* 1967). Therefore it was conceivable that, despite the precautions taken, enzymic breakdown of the 23s component had occurred. Further justification for this supposition was obtained from the fact that the relative proportion of this component diminished, in some experiments to an undetectable amount, when more time elapsed between the addition of the extracting mixture and the ultrasonic treatment stage. Substantially the same results were obtained when cells were pretreated with *m*-cresol and sodium benzoate, before ultrasonic treatment.

These experiments therefore established the presence of the 23s component of ribosomal RNA. However, it seemed likely that an enzyme was present that could hydrolyse the 23s component in such a way that the sedimentation coefficient of the hydrolysis products became similar to that of 16s ribosomal RNA. Though Lessie (1965) had

attempted to prove the absence of ribonuclease by adding *E. coli* ribosomal RNA to a cell-free extract of *R. sphaeroides*, the possibility was not excluded that a ribonuclease was bound to the ribosomes, possibly in close association with the 23s RNA component.

A possible interpretation of the results therefore is that this enzyme is activated, or its activity is largely enhanced, by lowering the viability of the cells before enzyme inhibitors could come into contact with the enzyme. That the enzymic breakdown takes place even in the actively growing cell cannot be excluded. The biological significance of this enzyme, if any, is not known. It may be pointed out that partial hydrolysis of the 23s RNA component of a ribosome may not necessarily cause the cessation of its function. Thus Jacob (1961) interpreted unpublished experimental results of Darnell to suggest that the ribosomal function in *E. coli* need not depend on the linear integrity of the RNA molecules within it. This conclusion followed from the observation that ribosomes in which 5–10 atoms of ³²P had decayed were still able to support phage protein synthesis in infected *E. coli* cells that were known to be unable to form new ribosomes.

It is further possible that, as suggested by Elson (1967) for *E. coli*, ribonuclease, which has been found to be associated with ribosomes in this organism, may have the function of initiating the degradation of ribosomal RNA in order to regulate the concentration of ribosomes within the cell.

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