

TETRACYCLINE INHIBITION OF *MYCOPLASMA* RIBOSOMAL PROTEIN SYNTHESIS

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Polyuridylic acid directed protein synthesis by ribosomes from tetracycline-sensitive and tetracycline-resistant cells of *Mycoplasma laidlawii* B was found to be sensitive to tetracycline. The ribosomal preparations were somewhat more sensitive to tetracycline than mixtures of ribosomes and cell membrane fragments. No requirement for an exogenous *m*-RNA could be demonstrated with the latter preparations.

In the past several years, the mechanism of protein biosynthesis in plant, animal and bacterial systems has been extensively investigated and at the present time is relatively well understood¹. The basic mechanisms in all these free-living forms appear to be essentially the same. In several brief reports²⁻⁴) TOURTELLOTTE *et al.* described cell-free protein synthesis in *Mycoplasma laidlawii* B in which similarities with other organisms were noted. They utilized a mixture of ribosomes and cell membranes in these studies, and the contributions of each of these components to the protein synthesis was not clearly defined. We have been successful in obtaining ribosomal fractions from *Mycoplasma* cells and have used them in studying the sensitivity of protein synthesis in this system tetracycline.

Materials and Methods

Materials

The following chemicals, biochemicals and their sources were used in these studies: L-Phenylalanine-U-¹⁴C, 374 mc/mM (New England Nuclear); adenosine triphosphate, guanosine triphosphate, phosphoenolpyruvate, pyruvate kinase, Tris buffer, and polyuridylic acid (all from Sigma Chemical Company); soluble ribonucleic acid (as sodium salt) (General Biochemicals Inc.); dithiothreitol and β -mercaptoethanol (Calbiochemicals); L-amino acids (Mann Research Laboratories); tetracycline hydrochloride (Lederle Laboratories).

Cultures

A culture of *Mycoplasma laidlawii* B was obtained from Prof. H. E. MORTON (University of Pennsylvania) and was maintained on PPLO agar supplemented with PPLO serum fraction (Difco). A mutant of this culture resistant to 50 μ g/ml tetracycline was obtained by repeated exposure of the parent (which is sensitive to 2 μ g/ml tetracycline). This mutant has maintained its resistance when transferred on media containing tetracycline.

Methods

Preparation of *Mycoplasma laidlawii* cells: Four 2-liter cotton-plugged flasks each containing 1-liter quantities of sterile tryptose broth (Tryptose (Difco), 20 g; NaCl, 5 g; Tris buffer, 3.7 g; glucose, 10 g; distilled water, q. s. 1 liter; pH adjusted with NaOH to pH 8.0~8.2 before autoclaving for 30 minutes at 121°C) were supplemented with 5 ml of

PPLO serum fraction (Difco) and inoculated with 50 ml of a 24-hour old culture of *M. laidlawii* B grown in the same medium. The flasks were incubated for 12 hours at 37°C (without shaking) and the cells collected by centrifugation at 5,000 rpm (3,000 × *g*) for 15 minutes at 4°C. The 1 g of collected cells was washed with 10 ml of chilled medium and centrifuged at 5,000 × *g* for 20 minutes at 4°C.

Preparation of ribosomes+cell membrane fragments: One gram of cells was suspended in 7 ml of 0.01 M pH 7.8 Tris buffer containing Mg(C₂H₃O₂)₂ (final concentration 0.01 M), KCl (final concentration 0.06 M), β-mercaptoethanol (final concentration 0.006 M) and crushed twice in a Aminco French press (4,000 lbs/sq. in.). The resulting paste was then homogenized in a glass Potter-Elvehjen homogenizer. The resulting slurry was utilized immediately without storage.

Preparation of ribosomes: Ribosome preparations free of membrane were obtained by taking 1 g of cell paste (obtained by the centrifuging mentioned above) suspended in 4 ml of 0.01 M pH 7.6 Tris buffer containing Mg(C₂H₃O₂)₂ (final concentration 0.016 M), KCl (final concentration 0.06 M), and dithiothreitol (final concentration, 0.1 mM). This suspension was placed in a 5 ml cup containing 3 ml of 120 μ glass beads attached to a Vibrogen cell mill (Model 600-00, manufactured by the Edmund Buhler Co.). The mill was operated at maximum speed for 30 minutes and the cup was maintained at 0°C by continuously passing chilled ethylene glycol through the jacket. The broken cell-bead mixture was then centrifuged at 18,000 × *g* for 30 minutes at 4°C and the supernatant solution dialyzed at 4°C overnight against the Tris buffer-Mg(C₂H₃O₂)₂-KCl-dithiothreitol solution. This dialyzed S-30 fraction was stored in small quantities at -20°C and activity was maintained for at least 10 days under these storage conditions.

Determination of protein synthesizing ability: The reaction mixture for the crushed cells+membranes contained: Tris buffer, pH 7.8, 100 μM; KCl, 50 μM; Mg(C₂H₃O₂)₂, 10 μM; β-mercaptoethanol, 6 μM; adenosine triphosphate, 1 μM; phosphoenolpyruvate, 5 μM; phosphoenolpyruvate kinase, 2 μg; the 20 amino acids commonly found in proteins (except for phenylalanine), 0.05 μM; L-phenylalanine-U-¹⁴C (sp. act. 187 μCi/μM) 0.1 μCi; L-phenylalanine, 26 μM; crushed cells (including membranes) 50 μg protein; total volume, 0.10 ml.

The reaction mixture for the ribosomes contained: Tris buffer, pH 7.6, 50 μM; Mg(C₂H₃O₂)₂, 18 μM; NH₄Cl, 50 μM; dithiothreitol, 0.1 μM; adenosine triphosphate, 1 μM; guanosine triphosphate, 0.5 μM; phosphoenolpyruvate, 5 μM; phosphoenolpyruvate kinase, 2 μg; polyuridylic acid, 10 μg; *t*-ribonucleic acid (from *Escherichia coli* B), 0.1 mg; L-phenylalanine-U-¹⁴C (specific activity 187 μCi/μM), 0.1 μCi; L-phenylalanine, 26 μM; total volume, 0.10 ml. In those reactions in which the S-30 fraction was used, there was approximately 0.1 mg protein added to the reaction mixture. In the other experiments where the ribosomes were separated from the supernatant solution, 0.09 mg ribosomes and 0.09 mg of supernatant protein were added to the reaction mixture.

The reaction mixtures were incubated for up to 60 minutes at 37°C and then chilled in ice water. One ml of distilled water and 0.05 ml of 10 % bovine serum albumin were added followed by 1.0 ml of 10 % trichloroacetic acid. The resulting suspension was heated at 100°C for 15 minutes, chilled, and the precipitate collected on a Millipore filter. The collected precipitate was washed with 5 % trichloroacetic acid, the filters dried and the absorbed radioactivity determined in a Packard TriCarb liquid scintillation spectrometer (model 2002) using BRAY's solution⁵.

Protein determination: The biuret method was used with crystalline bovine serum albumin as standard⁶.

Results

Experiments with Crushed Cells:

The incubation was performed as described above. The optimum conditions for

protein synthesis was found to be: 30 minutes incubation time; 37°C incubation temperature; about 10 μ M $Mg(C_2H_3O_2)_2$ per 0.1 ml reaction mixture; pH 7.8. No difference in protein synthesis was noted when polyuridylic acid was added to the mixture. The inhibition of protein synthesis noted when tetracycline

was added to the reaction mixture containing crushed cell fragments and ribosomes from both the tetracycline-sensitive *M. laidlawii* B and a tetracycline-resistant mutant is summarized in Table 1. Addition of tetracycline to give a final concentration of about 6×10^{-5} M decreased protein synthesis by more than 50% with both crushed cell preparations.

Experiments with Ribosomes

The procedure mentioned above was used with the ribosomes. A definite requirement was found for polyuridylic acid for protein synthesis by ribosomes from the tetracycline-sensitive and from the tetracycline-resistant *M. laidlawii*, and protein synthesis was inhibited by addition of tetracycline. Some of the data collected in this study are summarized in Table 2.

Table 1. Tetracycline inhibition of protein synthesis by crushed cells of *Mycoplasma laidlawii* B

Amount tetracycline present in reaction mixture	Counts/min. of protein by crushed cell preparations from:	
	Tetracycline-sensitive cells	Tetracycline-resistant cells
None	1293	949
6.2×10^{-5} M	249	326
6.2×10^{-6} M	937	814
6.2×10^{-7} M	1240	889

Table 2. Tetracycline inhibition of protein synthesis by ribosomes from *Mycoplasma laidlawii* B

Amount tetracycline present in reaction mixture	Counts/min. of protein formed by ribosomes from:			
	Tetracycline-sensitive cells		Tetracycline-resistant cells	
	System+ polyuridylylate	System without polyuridylylate	System+ polyuridylylate	System without polyuridylylate
None	2205	506	1965	935
6.2×10^{-5} M	601		955	
6.2×10^{-6} M	1136		1683	
6.2×10^{-7} M	2041		1854	

Discussion

Requirements for protein synthesis by *M. laidlawii* ribosomes:

Definite requirements for the 105,000 $\times g$ supernatant solution, ribosomes, adenosine triphosphate, phosphoenolpyruvate, phosphoenolpyruvate kinase, guanosine triphosphate, $Mg(C_2H_3O_2)_2$ (more than 13 μ M per 0.1 ml reaction mixture), and dialysis of the ribosomal fraction were demonstrated by elimination experiments. No requirement for cytosine triphosphate, uridine triphosphate, and the 20 common amino acids found in proteins (except for phenylalanine) could be demonstrated in these elimination experiments. In similar studies with the crushed cells no requirements for *t*-ribonucleic acid, polyuridylic acid, guanosine triphosphate, and $Mg(C_2H_3O_2)_2$ could be demonstrated.

Differences with previously reported studies:

The earlier studies by TOURTELLOTTE *et al.*²⁻⁴ showed that protein synthesis by crushed cell preparations (including membranes) required particulate materials from the cells. Endogenous incorporation of amino acids into protein occurred. It seems likely that the ribosomes were intimately involved with the cell membranes and for some reason poly-

uridylic acid could not bind to them. The Vibrogen cell mill prepared S-30 fractions of *Mycoplasma* are more active than the crushed cell preparations because no cell membranes are needed for high incorporations of phenylalanine into trichloroacetic acid precipitable protein.

Origin of tetracycline resistance in *Mycoplasma* :

It appears from the data summarized in Tables 1 and 2 that tetracycline resistance in *Mycoplasma* is due to some transport phenomenon with the cell membrane being the mediator of this process. If a ribosomal mechanism is involved we expect that the cell-free systems from the tetracycline-resistant mutant would be less sensitive to tetracycline than those from the tetracycline-sensitive parent culture.

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References

- 1) LENGYEL, P. & D. SÖLL: Mechanism of protein biosynthesis. *Bact. Rev.* 33 : 264~301, 1969
- 2) TOURTELLOTTE, M. E. & H. J. MOROWITZ: Cell-free protein synthesis in *Mycoplasma laidlawii*. *Bacteriol. Proc.* p. 112, 1963
- 3) TOURTELLOTTE, M. E.; M. E. POLLACK & R. P. MALEWAIK: Protein synthesis in *Mycoplasma*. *Ann. N. Y. Acad. Sci.* 143 : 130~138, 1967
- 4) TOURTELLOTTE, M. E.: Protein synthesis in *Mycoplasmas*. in *The Mycoplasmatales and the L-Phase of Bacteria* (LEONARD HAYFLICK, editor). pp. 451~468. New York: Appleton-Century-Crofts, 1969
- 5) BRAY, G. A.: A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1 : 279~285, 1960
- 6) LAYNE, E.: Spectrophotometric and turbidimetric methods for measuring proteins. in *Methods in Enzymology*. Volume III (S. P. COLOWICK & N. O. KAPLAN, editors). pp. 450~451. New York: Academic Press, Inc. 1957