Purification of 70S Ribosomes from *Bacillus subtilis*

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[Abstract] The eubacterial ribosome (70S) is a macromolecular complex that is composed of a small (30S) subunit and a large (50S) subunit. The small subunit comprises the 16S ribosomal RNA (rRNA) and more than 20 ribosomal proteins (r-proteins), whereas the large subunit comprises the 23S and 5S rRNAs and more than 30 r-proteins. *Bacillus subtilis* (*B. subtilis*) has 57 r-ribosomal protein genes and three rRNAs (16S, 23S and 5S rRNAs). Among them, we identified 21 r-proteins of the small subunit and 31 r-proteins of the large subunit in *B. subtilis* (Nanamiya *et al*., 2004). The functions and roles of individual components of the ribosome have not yet been completely clarified. Herein we describe in detail an ultracentrifugation-based protocol for the preparation of 70S ribosomes from exponentially growing cells of *B. subtilis*.

Materials and Reagents

1. *Bacillus subtilis* 168
2. NaCl
3. BD Bacto™ tryptone (Difco, catalog number: 211705)
4. BD Bacto™ yeast extract (Difco, catalog number: 212750)
5. Agar (Nissui, catalog number: 05835)
6. Tris (Wako Pure Chemical Industries, catalog number: 512-97505)
7. Magnesium acetate (Wako Pure Chemical Industries, catalog number: 139-15335)
8. Ammonium acetate (Wako Pure Chemical Industries, catalog number: 019-02835)
9. Dithiothreitol (DTT) (Wako Pure Chemical Industries, catalog number: 042-29222)
10. Phenylmethylsulphonyl fluoride (PMSF)
11. Sucrose (Sigma-Aldrich, catalog number: S0389-55G)
12. Diethylpyrocarbonate (DEPC) treated water
    Note: DEPC was added to distilled water at a final concentration of 1%, and then it stored at room temperature for over two hours. The DEPC-treated distilled water was autoclaved twice (121 °C, 20 min) and stored at room temperature.
13. LB medium (see Recipes)
14. Buffer I (see Recipes)

15. 10-40% sucrose solutions (see Recipes)

Equipment

1. 2 L flasks with cotton plugs
2. Innova 4080 bench top incubator shaker (New Brunswick scientific)
3. French pressure cell press [Aminco International (USA), model: FA-078]
4. Mini cell [Aminco International (USA), model: FA-003]
5. Gradient fractionator (BIOCOMP, catalog number: 152-001)
6. Gradient master [Aminco International (USA), catalog number: 107-201M]
7. Micro collector (ATTO Technology, catalog number: AC-5700P)
8. Nano drop 2000 (Thermo Fisher Scientific)
9. High-speed refrigerated centrifuge (*e.g.* Hitachi, model: himac CR22GII)
10. Angle rotor R10A3 (fixed angle, max: 10,000 rpm,18,800 *x g*, volume: 500 ml x 6) (Hitachi)
11. Centrifuge MX-300 (TOMY)
12. TMA-300 rotor (fixed angle, max: 15,000 rpm, 20,380 *x g*, volume: 2 ml x 24) (TOMY)
13. TMA-27 rotor (fixed angle, max: 15,000 rpm, 21,130 *x g*, volume: 15 ml x 4, 50 ml x 4) (TOMY)
14. Centrifuge tube (Nalgene, catalog number: 3148-0050)
15. Ultra-centrifuge (*e.g.* Hitachi, model: CP-60E)
16. P55ST2 rotor (swinging bucket rotor, max: 55,000 rpm, 366,000 *x g*, volume: 5 ml x 6) (Hitachi)
17. P28S rotor (swinging bucket rotor, max: 28,000 rpm, 141,000 *x g*, volume: 40 ml x 6) (Hitachi)
18. P40ST rotor (swinging bucket rotor, max: 40,000 rpm, 284,000 *x g*, volume: 13 ml x 6) (Hitachi)
19. Open-top polyclear centrifuge tube for P40ST rotor (Seton Identification Products, part number: 7031)
20. Open-top polyclear centrifuge tube for P28S rotor (Seton Identification Products, part number: 7052)
21. Centrifuge ware for P55ST2 rotor (Hitachi, part number: 332245A)
    Note: These rotors can substitute for other commercial rotors which can be used by the same centrifugal force as described in procedures.

Procedure

A. Crude preparation of 70S ribosomes

1. *B. subtilis* is streaked out for single colonies and pre-incubated on LB agar plates at 30 °C for 14-16 h.
2. Cells are taken from single colonies on the plates and suspended in approximately 10 ml of LB medium with high turbidity. The cell suspension was diluted into 3 L of LB medium at an OD_{600} of 0.04, and distributed equally among six 2-liter flasks, each containing 500 ml of the cell culture. The flasks are incubated at 37 °C with shaking at 250 rpm.

   Note: B. subtilis is routinely cultured in volumes that do not exceed one quarter of the volume of the flask used.
3. Cells are harvested by centrifugation, when the OD_{600} of the culture reaches 0.2, using six 500-ml centrifuge tubes (7,000 x *g*; 5 min, 4 °C). Cell pellets are resuspended with small volume of the culture supernatant, transferred from a 500-ml centrifugation tube to six 15-ml sample tubes and pellets are collected (3,500 x *g*, 5 min, 4 °C). The cell pellets are frozen by liquid N_2.
4. Each cell pellet is suspended in 3.5 ml of Buffer I pre-chilled on ice.
5. Cells are disrupted using a French press (8,000 p.s.i.) passing the sample through three times.
6. Cell debris is removed by centrifugation (11,000 x *g*, 30 min, 4 °C).
7. The supernatant (cell lysate) is centrifuged at 30,000 x *g* for 30 min at 4 °C, for example in a HITACHI P55ST2 rotor at 18,000 rpm.
8. The resulting supernatant (S30 supernatant) is centrifuged at 200,000 x *g* for 100 min at 4 °C (*e.g.* in a HITACHI P55ST2 rotor at 45,000 rpm).
9. Each pellet is resuspended with 200 µl of Buffer I and the suspension stored at -80 °C until required (S100 pellet fraction).

   Note that the S100 fraction can be seen as amber pellet at bottom of the tube. Using a glass rod may be preferable to resuspend the pellet because resuspension of the pellet by pipetting is not easy.

   Note: Care is taken to avoid generating foam while resuspending the pellet.

B. Purification of 70S ribosomes

10. To further purify the 70S ribosomes, prepare a 10-40% sucrose gradient in SW28 centrifuge tubes using a Gradient Master gradient mixer.
11. 10% and 40% sucrose solutions are prepared in Buffer I.
12. The 10 to 40% sucrose gradient is generated in a SW28 centrifuge tube using a Gradient Master gradient mixer.
13. The S100 pellet fraction is applied on the top of the 10 to 40% sucrose gradient and centrifuged at 67,000 x *g* for 17 h at 4 °C (*e.g.* in a HITACHI P28S rotor at 22,500 rpm, using 270 A_{260} units per centrifuge tube).

14. Samples are taken by Piston Gradient Fractionator and Micro collector. Absorbance profiles at 260 nm are determined using a Nano drop 2000.
    Note: An example of the ribosome profile from crude cell extracts is shown in Figure 1.
15. Collected fractions containing 70S ribosomes are identified as a peak with an absorbance at 260 nm.
16. The ribosomes are recovered from the pooled fractions, which are diluted with Buffer I (1:2 dilutions) and centrifuged at 64,000 x *g* for 17 h at 4 °C. (*e.g.* in a HITACHI P40ST rotor at 22,500 rpm).
17. The supernatant is removed completely from the centrifuge tubes.
18. The resulting 70S ribosome pellets are covered with 300 µl of Buffer I and left overnight at 4 °C. The pellet is then gently resuspended in Buffer I using a glass rod and stored at -80 °C until required.
    Note: Care is taken to avoid generating foam while resuspending the pellet.

Representative data

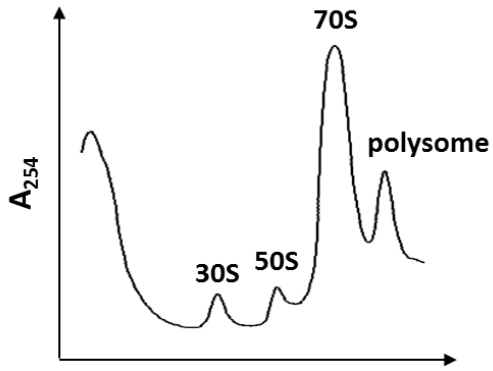

Figure 1. Profile of ribosome from crude cell extract by 10-40% sucrose gradient sedimentation. The crude cell extract prepared from the log phase cells (OD_{600} = 0.2) of wild type strain (168 *trpC2*) was applied on the top of the 10 to 40% sucrose gradient and centrifuged at 55,000 x g for 17.5 h at 4 °C (*e.g.* in a HITACHI P40ST rotor at 21,000 rpm, using 10 A_{260} units per centrifuge tube). The A_{260} measurement shows as the same profile pattern of A_{420} measurement, and the peak of 70S ribosome can be determined. When the S100 fraction was subjected to the 10 to 40 % sucrose gradient and centrifugation, the peak smaller than 30S was not observed.

Recipes

1. LB medium
   0.5% NaCl

1% tryptone
0.5% yeast extract
1.5% agar (only use for plates)

2. Buffer I (200 ml is enough, pre-chilled on ice)
   20 mM Tris-HCl (pH 7.6)
   15 mM magnesium acetate
   100 mM ammonium acetate
   0.1 mM DTT
   2 mM PMSF
   Buffer I is prepared with DEPC treated water.
   Note; Buffer I can be stored without DTT and PMSF. DTT and PMSF are added to the buffer before use. Buffer I should be pre-chilled throughout the entire procedure.
3. 10-40% sucrose solutions
   10-40% sucrose solutions are made in Buffer I.

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