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A single-step method for purification of active His-tagged ribosomes from a genetically engineered *Escherichia coli*

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ABSTRACT

With the rapid development of the ribosome field in recent years a quick, simple and high-throughput method for purification of the bacterial ribosome is in demand. We have designed a new strain of *Escherichia coli* (JE28) by an in-frame fusion of a nucleotide sequence encoding a hexa-histidine affinity tag at the 3′-end of the single copy *rplL* gene (encoding the ribosomal protein L12) at the chromosomal site of the wild-type strain MG1655. As a result, JE28 produces a homogeneous population of ribosomes $(His)_6$-tagged at the C-termini of all four L12 proteins. Furthermore, we have developed a single-step, high-throughput method for purification of tetra-$(His)_6$-tagged 70S ribosomes from this strain using affinity chromatography. These ribosomes, when compared with the conventionally purified ones in sucrose gradient centrifugation, 2D-gel, dipeptide formation and a full-length protein synthesis assay showed higher yield and activity. We further describe how this method can be adapted for purification of ribosomal subunits and mutant ribosomes. These methodologies could, in principle, also be used to purify any functional multimeric complex from the bacterial cell.

INTRODUCTION

The ribosome, comprising at least 50 proteins and three RNAs (5S, 16S and 23S), is the largest macromolecular assembly of the bacterial cell. Recent breakthroughs in the structural studies with bacterial ribosome (1–3), have shifted the major emphasis of the ribosome field towards further elucidation of the structure–function relationships. At the same time there is a growing interest in the cell-free system reconstituted from the components of the cellular transcription–translation machinery for the custom synthesis and window labeling of proteins and peptides (4,5), which has ribosome as the major component. Most of these studies rely on the purification of active ribosomes from the bacterial cells, more specifically from *Escherichia coli*, which is most widely used for the basic research on bacterial protein synthesis (3,6,7), visualization of ribosomal complexes by cryo-EM (3,6,8) and for reconstituted *in vitro* translation systems (3–5). Conventional method of *E. coli* ribosome purification (9) involves several steps of ultracentrifugation and/or column chromatography, and is therefore quite expensive in terms of time, effort, equipment and reagents. A simple, high-throughput method for purification of functional ribosome from *E. coli* is therefore in demand.

Affinity-tag-based purification method has revolutionized the protein purification field. Naturally, attempts have been made to purify bacterial, plant and yeast ribosomes using affinity tags (10–15). Two of these methods employed streptavidin-binding aptamer tag (12) and MS2 coat protein-binding tag (13) respectively, fused with the rRNA operon on a plasmid. Both of these methods were designed mainly for the purification of *E. coli* ribosome-bearing mutations in the rRNAs. The other methods involved fusion of either Flag-$(His)_6$ tag (11,14) or S-peptide tag (10) to some ribosomal protein from *Saccharomyces cerevisiae* and *Arabidopsis thaliana* respectively, over-expressed from a plasmid. Since all of these methods employ plasmid based over-expression of a ribosomal component fused with the affinity tag the success of these methods depends on the level of over-expression and also on the efficiency of assembly of the over-expressed tagged component onto the ribosome. The yield of the tagged ribosomes in these cases varies from 4% (15) to 40% (13). Another standing problem of these systems is that the tagged protein or the RNA component, over-expressed from the plasmids, is usually produced in huge

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excess over the ribosomes, thus demanding additional purification steps for their separation. Therefore, a more efficient and high-yield system for affinity-tag-based purification of the active bacterial ribosomes was somewhat lacking and needed to be developed. Also, from the perspective of the bacterial physiology the presence of the normal ribosomes together with the tagged ones made the impact of the tag insertion upon *in vivo* translation and growth rate unclear.

As a solution to the above-mentioned limitations we have constructed a novel *E. coli* strain JE28, in which a $(His)_6$-tag has been inserted at the C-terminus of the ribosomal protein L12 engineering directly on the chromosome, using lambda (λ) Red recombineering (16,17). Since L12 is present in four copies on the large subunit of *E. coli* ribosome (18,19), all ribosomes of JE28 are homogeneously tetra-$(His)_6$-tagged. Next, we developed a single-step, affinity chromatography-based, fast and easy method for the purification of the tetra-$(His)_6$-tagged ribosomes from this strain, which is essentially identical to the purification of any His-tagged protein. This method can be easily modified for the purification of ribosomal subunits and any mutant *E. coli* ribosome. The JE28 ribosomes purified in this method are characterized and compared with the wild-type ribosomes purified in the conventional way.

MATERIALS AND METHODS

Preparation of linear DNA cassette for λ Red recombineering

Standard PCR conditions were used to amplify the kanamycin-resistant cassette (*kan*) using pET-24b plasmid (Novagen) as a template and two specially designed primers (Figure 1a). The forward primer used for PCR had the sequence 5′-GAAAAAAGCTCTGGAAGAAGCTGGC GCTGAAGTTGAAGTTAAACACCACCACCACCAC CACTAAAAACAGTAATACAAGGGGTGTTATG-3′, that contained 43 nucleotides homologous to the 3′-end of the *E. coli rplL* gene (coding for ribosomal protein L12) minus the stop codon, followed by six CAC repeats coding for six histidines, then stop codon TAA and at last 25 nucleotides homologous to the beginning of the *kan* cassette on the Novagen pET-24b plasmid. The reverse primer (5′-ATCAGCCTGATTTCTCAGGCTGCAACC GGAAGGGTTGGCTTAGAAAAACTCATCGAGCA TCAAATGAAA-3′) contained sequences, reverse complementary to 39 nucleotides located immediately after the *rplL* gene followed by the reverse complementary sequence to last 30 nucleotides of the *kan* cassette of pET-24b. Both the primers were purchased from Invitrogen as custom synthesized and PAGE purified. The PCR product was purified from agarose gel using a commercial kit (Qiagen) and was used as linear DNA cassette for λ Red recombineering.

Construction of the *E. coli* strains JE5 and JE28

Strain JE5 was constructed from *E. coli* HME6 strain [W3110 *Δ(argF-lac)U169 gal*$^+$ {λcI857Δcro-bioA} *galK*$_{TYR145UAG}$] (16,17), where the stop codon of the *rplL* gene was replaced by a linear PCR product encoding six histidines, a TAA stop codon followed by kanamycin-resistance cassette (*kan*), using the λ Red recombineering system (20,21) (Figure 1b). HME6 cells were made recombinogenic and electroporation-competent following a previously described protocol (21). One to two microliters of

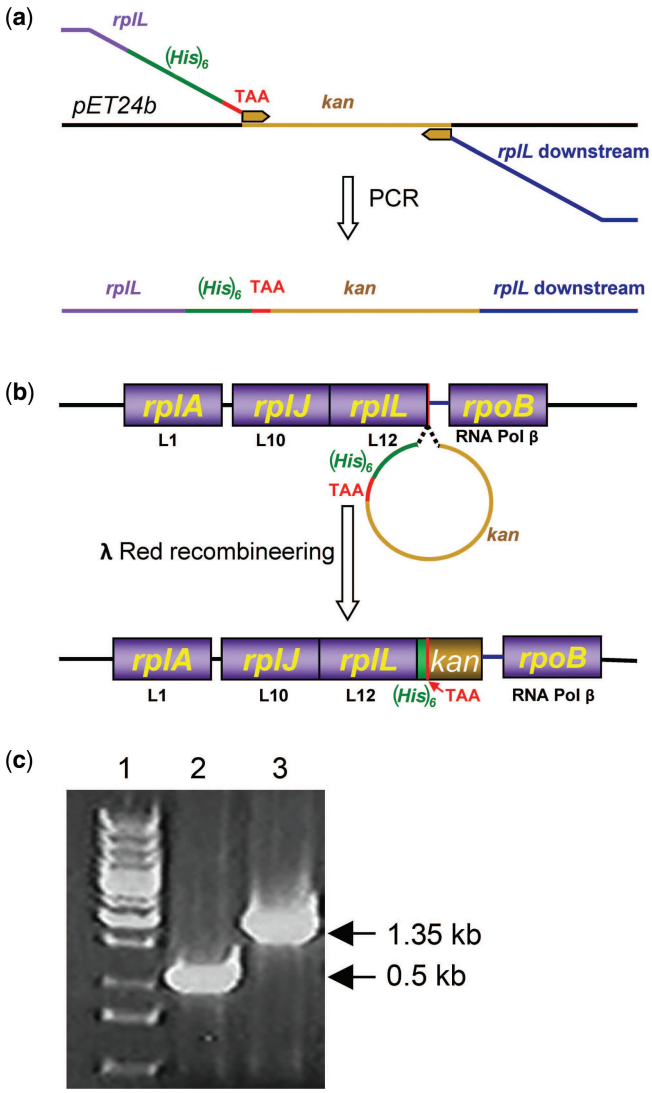

Figure 1. The chromosomal insertion of a nucleotide sequence encoding six histidines at the 3′-end of the *rplL* gene followed by a *kan* marker and its verification. (**a**) A linear DNA (856 bp) was synthesized by PCR amplification of kanamycin-resistant cassette (*kan*) from pET-24b plasmid (Novagen). The sequences needed for homologous recombineering at the *rplL* site, as well the sequence encoding $(His)_6$ and a stop codon TAA were introduced by specially designed primers described in the Materials and methods section. (**b**) The linear DNA cassette encoding $(His)_6$, a stop codon and *kan* was inserted by λ Red recombineering, at the 3′-end of the *rplL* gene at the chromosomal site of *E. coli* HME6. The resulting strain was named JE5 [HME6 (*rplL-his*$_6$):*kan*:*rpoB*$^+$]. Next, the *kan* linked, His-tagged *rplL* gene was transferred from JE5 to the wild-type *E. coli* strain MG1655 by generalized transduction with bacteriophage P1(22) yielding a new stable *E. coli* strain JE28 [MG1655 (*rplL-his*$_6$):*kan*:*rpoB*$^+$]. (**c**) Verification of the linear cassette insertion by colony-PCR using primers flanking *rplL* region; lane 1: mol. wt. marker; lane 2: wild-type MG1655; lane 3: JE5. The JE5 as well as JE28 (data not shown) colony-PCR produced a band about 850 bp bigger than that from MG1655 indicating successful insertion of the DNA cassette.

high-quality PCR product (200–400 ng/μl) was added to 100 μl electro-competent HME6 cell suspensions in H_2O and electroporated at 1.8 kV, 25 μF and 200 Ohms. The electroporated cells were incubated overnight in 1 ml LB at 30°C with aeration. Successful chromosomal recombinant colonies were selected on kanamycin plates and were confirmed by PCR with primers homologous to the flanking regions of the target site (Figure 1c). We also sequenced the 3′-end of the *rplL* gene from some of the recombinant colonies to confirm the correct insertion of the $(His)_6$-tag at the C-terminus of L12. The ones with the correct insertion were named JE5 [HME6 (*rplL-his*$_6$):*kan*:*rpoB*$^+$]. Furthermore, the *kan*-linked, His-tagged *rplL* gene was transferred from JE5 to the wild-type *E. coli* lab-strain MG1655 by generalized transduction with bacteriophage P1 (22) yielding a new stable *E. coli* strain JE28 [MG1655 (*rplL-his*$_6$):*kan*:*rpoB*$^+$]. JE28 strain carried kanamycin resistance, produced a PCR product identical to JE5 and the sequencing of the 3′-end of the *rplL* gene confirmed the presence of an in-frame insertion of the $(His)_6$-tag at the C-terminus of L12 protein.

Measurement of the growth-rate

The growth rate of JE28 (with and without 50 μg/ml kanamycin, respectively) was compared with that of its parental strain MG1655 in LB at 37°C, by following the absorbance at 600 nm with time.

Purification of the tetra-$(His)_6$-tagged ribosomes from *E. coli* JE28

A fresh O/N culture of *E. coli* JE28 was used to inoculate 1 l. LB with 50 μg/ml kanamycin and grown with aeration at 37°C. At A_{600} 1.0, the culture was slowly cooled to 4°C to produce run-off ribosome and harvested by centrifugation at 4000 rpm for 30 min. The cell-pellet was resuspended in lysis buffer (20 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl and PMSF protease inhibitor 200 μl/l) with lysozyme (0.5 mg/ml) and DNAse I (10 μg/ml) and further lysed using a French Press or sonicator (for smaller cell pellets <2–3 g). The lysate was clarified by centrifuging twice at 18 000 rpm at 4°C, 20 min each. The cleared lysate was divided in half. From one-half 70S ribosome was purified in the conventional method and the affinity-purification method was employed with the other half. In parallel, wild-type ribosome was also purified from the parent strain MG1655 in the conventional way for comparison.

For *affinity purification*, a HisTrap^{TM}HP column (Ni^{2+}–sepharose pre-packed, 5 ml, GE Healthcare) was connected to an ÄKTA prime chromatography system (GE Healthcare) equilibrated with the lysis buffer. After loading the lysate, the column was washed with 5 mM imidazole until A_{260} reached the baseline. The tetra-$(His)_6$-tagged ribosomes were then eluted with 150 mM imidazole, pooled immediately and dialyzed 4× for 10 min in 250 ml lysis buffer to remove the imidazole. Furthermore, the ribosomes were concentrated by centrifugation at 150 000 × *g* for 2 h at 4°C, resuspended in 1× polymix buffer containing 5 mM ammonium chloride, 95 mM potassium chloride, 0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate and 1 mM dithioerythritol (23) and shock-frozen in liquid nitrogen for storage or dissolved in the overlay buffer (20 mM Tris–HCl pH 7.6, 60 mM NH_4Cl, 5.25 mM Mg acetate, 0.25 mM EDTA and 3 mM 2-mercaptoethanol) for sucrose gradient analysis. As a control, lysate from wild-type *E. coli* MG1655 was applied to the same column and was treated accordingly.

For purifying JE28 and MG1655 ribosomes in the conventional *ultracentrifugation method* (24), the cleared lysate was layered on top of equal volume of 30% w/v sucrose cushion made in a buffer containing 20 mM Tris–HCl pH 7.6, 500 mM NH_4Cl, 10.5 mM Mg acetate, 0.5 mM EDTA, and 7 mM 2-mercaptoethanol and centrifuged at 100 000 × *g* for 16 h at 4°C. This step was repeated twice and in between the ribosome pellet was gently rinsed with the same buffer. Then the pellet was dissolved in 1× polymix buffer for storage or in the overlay buffer for sucrose gradient analysis as in case of the affinity-purified ones.

Sucrose density-gradient analysis of tetra-$(His)_6$-tagged ribosomes from JE28

The tetra-$(His)_6$-tagged ribosome from JE28 purified by the affinity method was analyzed for the subunit composition by sucrose gradient centrifugation (9). Nearly 3000 pmol of ribosome was loaded on a 20–50% sucrose density gradient (18 ml) prepared in a buffer containing 20 mM Tris–HCl pH 7.6, 300 mM NH_4Cl, 5 mM Mg Acetate, 0.5 mM EDTA and 7 mM 2-mercaptoethanol and centrifuged at 100 000 × *g* for 16 h at 4°C. For comparison, JE28 ribosomes prepared in the ultracentrifugation method were also analyzed in parallel. We have used wild-type *E. coli* (MG1655) ribosome and subunits prepared in the conventional way (24) as standards.

2D-gel analysis of the JE28 tetra-$(His)_6$-tagged ribosome

2D-gel analysis of the purified ribosome from JE28 was done following the protocol of Geyl *et al.* (25) with minor modifications (9). As a control, 70S ribosomes from MG1655 were also subjected to similar analysis.

Activity of the purified ribosome in dipeptide formation assay

The dipeptide formation assay is an assessment of the peptidyl transferase activity of the ribosome. For the formation of dipeptide fMet–Leu, the protocol for the fMet–Phe dipeptide formation as described by Antoun *et al.* (26) was closely followed with necessary modifications. The components needed for the modification included an mRNA coding for fMet–Leu–Stop, tRNA aminoacyl synthetase LeuRS, $tRNA^{Leu}$ and the amino-acid Leu, instead of fMet–Phe–Thr–Ile–Stop mRNA, PheRS, $tRNA^{Phe}$ and the amino-acid Phe, respectively (26,27). Also, we replaced the LS-buffer (26) by 1× polymix buffer (23).

Cell-free synthesis of m-Cherry fluorescent protein

To check the activity of the tetra-$(His)_6$-tagged affinity-purified ribosomes from JE28 in all steps of protein synthesis, a full-length fluorescent protein, m-Cherry (28) was synthesized *in vitro* in a reconstituted coupled transcription–translation system. In this system we used a PCR-amplified DNA template for m-Cherry (28), T7-RNA polymerase (Sigma), purified ribosomes, translation factors and amino-acyl synthetases together with an 'energy mix' and amino-acid mix (Promega) and the reaction was conducted in 37°C directly in a quartz cuvette. The synthesis of m-Cherry was monitored by measurement of its fluorescence at 610 nm (Excitation at 500 nm). For comparison, ribosomes from the wild-type strain MG1655, purified in the conventional way, were also subjected to the same assay.

Purification of ribosomal subunits from JE28 with affinity chromatography

For purification of the ribosomal subunits employing *affinity method* the tetra-$(His)_6$-tagged ribosomes were dialyzed or diluted in low-Mg^{2+} buffer containing 20 mM Tris–HCl pH 7.6, 1 mM $MgCl_2$, 150 mM KCl and 30 mM NH_4Cl and was applied to a HisTrap^{TM}HP column equilibrated with the same buffer. Since the tera-$(His)_6$-tag was on the 50S subunit, the free 30S subunits were not retained on the column and were collected in the flow-through. The tetra-$(His)_6$-tagged 50S subunits were subsequently eluted from the column and the subunits were concentrated following the same procedure as described above for the $(His)_6$-tagged 70S ribosome.

For separation of ribosomal subunits in the *conventional way*, 70S ribosome was dialyzed in low-Mg^{2+} buffer containing 20 mM Tris–HCl pH 7.6, 300 mM NH_4Cl, 1 mM Mg Acetate, 0.5 mM EDTA, and 7 mM 2-mercaptoethanol and separated by ultra-centrifugation ($85\,000 \times g$ at 4°C for 16 h) on 20–50% sucrose density gradients (18 ml) prepared in the same (low-Mg^{2+}) buffer. The gradients were fractionated monitoring the absorbance at 260 nm. Respective peak fractions for 50S and 30S were pooled, concentrated by centrifugation at $150\,000 \times g$ for 2 h at 4°C, resuspended in 1× polymix buffer and stored in the same as described above for 70S ribosome.

Re-association of JE28 ribosomal subunits on the HisTrap^{TM}HP column

The 30S subunits isolated in the method described in the previous section were dialyzed in a high-Mg^{2+} buffer containing 20 mM Tris–HCl pH 7.6, 1 mM $MgCl_2$, 150 mM KCl and 30 mM NH_4Cl and the HisTrap^{TM}HP column with tetra-$(His)_6$-tagged 50S subunits bound on it was equilibrated with the same buffer. The 30S subunits were loaded slowly on the column and then the ribosomes were eluted with 150 mM imidazole. The eluted ribosomes were concentrated and subjected to sucrose gradient analysis to check the extent of on-column subunit re-association.

RESULTS AND DISCUSSION

JE28 [MG1655 (*rplL-his$_6$*):*kan*:*rpoB*$^+$] is a stable *E. coli* strain

The *E. coli* strain JE28 derived from the wild-type *E. coli* MG1655 is the first example of in-frame fusion of a nucleotide sequence encoding a hexa-histidine affinity tag with a *rpl* (ribosomal protein) gene at its chromosomal site together with a *kan* cassette as a selective marker. The site of the insertion is the 3′-end of the single copy *rplL* gene coding ribosomal 'stalk' protein L12 [also known as L7/L12 in *E. coli* (29) (Figures 1a and b)]. The total length of the inserted sequence is about 850 nucleotides. We have successfully grown JE28 without kanamycin selection on LA plates for several generations; the stability of the inserted sequence is confirmed by the retention of kanamycin resistance as well as maintenance of the intact *rplL* gene as checked by PCR using flanking primers (Figure 1c). When compared with MG1655, JE28 showed essentially the same growth rate in liquid culture (LB) (Figure 2); presence of kanamycin (50 μg/ml) in the medium did not have any effect on it (data not shown). Two important conclusions can be drawn from this result. (i) The targeted insertion at the chromosomal site of the *rplL* gene is stable and does not affect the expression of the genes located downstream on the *rplJL-rpoBC* operon (e.g. *rpoB* coding for RNA polymerase beta, see Figure 1b). (ii) The $(His)_6$-tags inserted on the C-termini of the L12 proteins have no negative effect on the function of the protein L12 and hence on the ribosomes *in vivo*. This has been tested later *in vitro*.

The C-terminus of the L12 protein is a perfect site for the insertion of the affinity tag on the ribosome

To develop an efficient system for affinity-tag-based ribosome purification the choice of the tag insertion site is

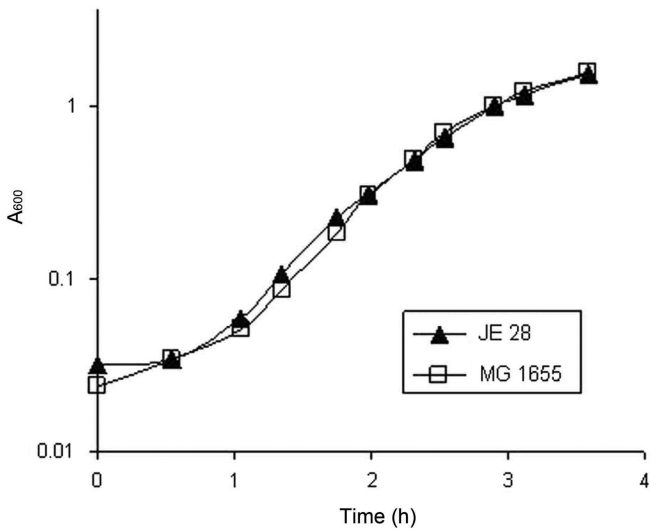

Figure 2. Comparison of the growth rates. The recombineered *E. coli* strain JE28 (filled triangle) shows no difference in growth rate (monitored by the absorbance at 600 nm) from the wild-type MG1655 (open square), when grown in LB at 37°C.

crucial. The C-terminus of the L12 protein was chosen for this purpose for several reasons. First, L12 is the only ribosomal protein present in multiple copies (19,29,30). In *E. coli*, there are four copies of L12 proteins on the 50S subunit (18). Therefore the chromosomal insertion of the nucleotide sequence encoding $(His)_6$-tag in the 3′-end of the *rplL* gene resulted in four $(His)_6$-tags on the JE28 ribosomes, which ensured its stable, specific and strong binding to the affinity matrix (Ni^{2+}–sepharose pre-packed HisTrap^{TM}HP column, GE Healthcare), resulting in very high yield of the ribosomes with high purity. In fact, similar fusion of $(His)_6$-tag with three other single copy ribosomal proteins L30, S2 and S3 were also successful, but none of those could yield ribosome of comparable quantity or purity using the same methodology.

The other reason for the choice of this site for the insertion was mainly structural. The protein L12, consists of three distinguishable domains; namely (i) N-terminal domain (NTD), involved in L12-dimer formation which binds to the C-terminus helix of the L10 protein (18), (ii) a globular C-terminal domain (CTD), highly conserved and exposed out of the ribosome, engaged in interactions with the translational factors (31–33) and (iii) a flexible hinge region connecting these two domains (18,30). Two dimers of the L12 proteins constitute a finger like protrusion from the 50S subunit called the ribosomal 'stalk' (30). Since the C-terminal domains of L12 dimers are in exposed conformation they provide very suitable sites for affinity-tag fusion (18,34), a feature essential for easy accessibility of the affinity tags to the matrix. Moreover, a recent NMR-study (33) has identified the factor-binding site on the L12 CTD, which is not the absolute end of the C-terminus. Therefore the fusion of a small $(His)_6$-tag in that site did not interfere with the L12 and hence ribosome function in the 'stalk'–factor interactions.

Affinity purification of tetra-$(His)_6$-tagged ribosome from *E. coli* JE28

A novel method for the purification of tetra-$(His)_6$-tagged ribosomes from *E. coli* JE28 is developed using affinity chromatography (see Materials and methods section for details). Figure 3a describes the elution profile of the tetra-$(His)_6$-tagged ribosome from the HisTrap^{TM}HP column (GE healthcare). The absorbance at 260 nm was monitored. The peak fractions eluted with 150 mM imidazole showed an A_{260}/A_{280} ratio of 1.9, a value typical for pure ribosomes (9). These fractions were pooled, concentrated and subjected to further analysis by sucrose density gradient centrifugation, 2D-gel and activity assay in peptide bond formation as well as by synthesis of a fluorescent protein mCherry (28). In a control experiment, where cleared cell lysate from wild-type *E. coli* MG1655 was applied to the HisTrap column, no ribosome was retained on the column.

Imidazole versus histidine

There are some previous reports of hydrolysis of RNA from prolonged exposure (several hours) to a somewhat high-concentration (1 M or above) of imidazole and bis-imidazole (35,36). Although the tetra-$(His)_6$-tagged ribosomes eluted from the HisTrap^{TM}HP column by 150 mM imidazole solution was found to be intact and active, histidine solution was tried as an alternative eluting reagent. When eluted with 100 mM Histidine the ribosome peak was somewhat broader than the imidazole-eluted peak and the yield was about 20% less (data not shown). It is also noteworthy that the rather expensive HisTrap^{TM}HP column could not be reused after one time elution with histidine without recharging with Ni^{2+}, but for imidazole elution the same column could be used several times.

Sucrose gradient analysis of the affinity-purified ribosome from *E. coli* JE28

The JE28 ribosomes purified by the affinity method were subjected to sucrose density-gradient centrifugation

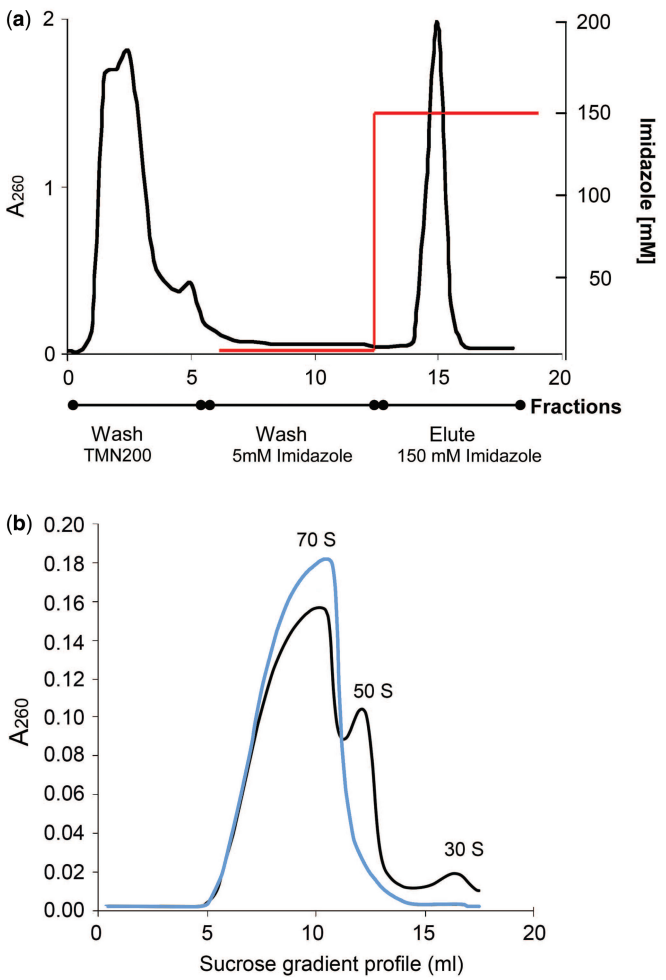

Figure 3. The purification and sucrose gradient centrifugation analysis of the tetra-$(His)_6$-tagged ribosome from *E. coli* JE28. (**a**) The cell-lysate of JE28 was loaded on a HisTrap^{TM}HP Ni^{2+}–sepharose column and the tetra-$(His)_6$-tagged ribosome was eluted with 150 mM imidazole (red line). (**b**) Sucrose density-gradient analysis of the JE28 ribosomes obtained by affinity purification (blue line) and conventional method (black line). The affinity-purified ribosomes comprised only 70S where as the conventional prep contained whole (70S) ribosomes as well as some subunits (50S and 30S).

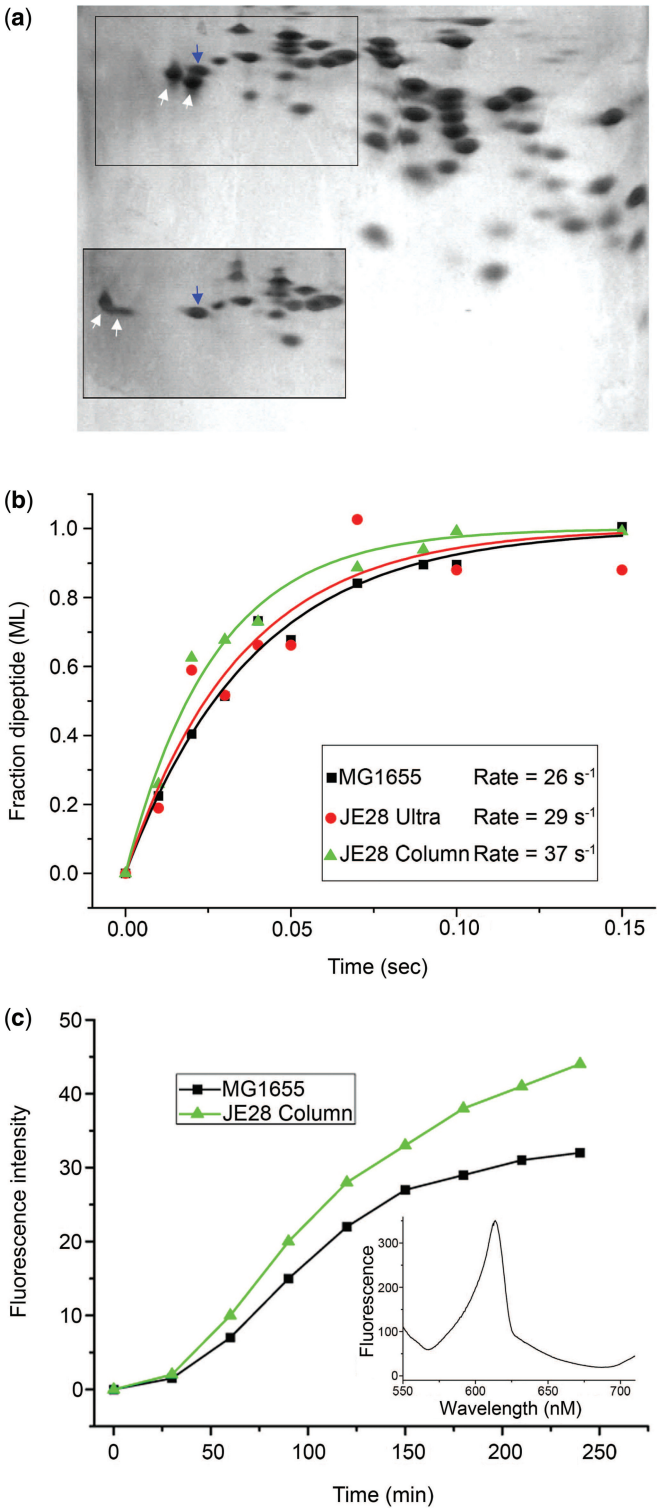

Figure 4. Characterization of the tetra-$(His)_6$-tagged ribosome from *E. coli* JE28. (**a**) 2D-gel analysis of the affinity-purified JE28 ribosomes showing protein L12 (or L7/L12 in *E. coli*) with white arrows and L10 with blue arrow. The relevant region is boxed and the inset (lower box) shows the same region from MG1655 ribosomes. (**b**) Activity of the affinity-purified JE28 ribosome (filled triangle, labeled as JE28 Column) in dipeptide-formation assay in comparison with MG1655 (filled square) and JE28 ribosomes purified in conventional ultracentrifuge method (filled circle, labeled as JE28 Ultra). An elongation mix containing Leu-ternary complex was added to a pre-incubated initiation complex and the amount of dipeptide (fMetLeu) formed at different times was monitored by HPLC. (**c**) The time course of the synthesis of mCherry fluorescent protein in a coupled transcription–translation system using affinity-column-purified JE28 (filled triangle), and the conventional ultracentrifugation purified MG1655 (filled square) ribosomes, monitored by the increase of its characteristic fluorescence at 610 nm. The inset shows the fluorescence spectra of the mCherry protein after 4 h of synthesis with affinity-purified JE28 ribosomes.

analysis together with those purified in the untracentrifugation method (see Materials and methods section). In the buffer conditions described in this work, the affinity-purified JE28 ribosomes contained only 70S, whereas ribosomes purified in the conventional way contained 50S and 30S subunits in addition to 70S (Figure 3b). The exact reason for this difference is not clear, but it resulted in a higher yield of 70S. It is possible that the much faster (1–2 h) affinity-purification method with fewer steps offers a relatively gentle treatment to 70S causing less disruption and dissociation of the loosely-coupled subunits, compared to the conventional method, which consumes much longer time (3–4 days), and involves several ultracentrifugation steps (24). Alternatively, it is also possible that during incubation and column-run in the high-Mg^{2+} buffer (10 mM), free 30S subunits associated with the 50S subunits fixed with the column matrix to form whole 70S.

2D-gel analysis of the affinity-purified ribosomes from *E. coli* JE28

The tetra-$(His)_6$-tagged JE28 70S ribosomes purified on a HisTrap^{TM}HP column were characterized in a 2D-gel (Figure 4a). In parallel, 70S ribosomes from MG1655 were also subjected to 2D-gel analysis for comparison (Figure 4a, inset). All the ribosomal proteins were identified in identical positions in both the samples with the exception of acidic L12 proteins (white arrow in Figure 4a), which were displaced from their original position towards another protein L10 (blue arrow in Figure 4a) due to the fusion of the $(His)_6$-tags that changed their pI from 4.6 to 5.2. It is worth mentioning that L12 appears as two connected spots on 2D-gel due to its L7 variant, which is the N-terminal acetylated form of L12. There is one additional protein-spot visible in the 2D-gel with JE28 ribosomes. This can be a truncated ribosomal protein. Its exact identity remains to be clarified.

In vitro activity assay of the affinity-purified JE28 ribosome-dipeptide formation and the synthesis of mCherry (28) fluorescent protein

Peptide bond formation is central to ribosome functions. In a cell-free translation system composed of purified components from *E. coli* (26,37), tetra-$(His)_6$-tagged JE28 ribosome purified in the affinity method showed a faster rate of dipeptide (fMet–Leu) formation ($37\,s^{-1}$) when compared to that of the JE28 ($29\,s^{-1}$) as well as MG1655 ($26\,s^{-1}$) ribosomes purified in the ultracentrifugation method (Figure 4b). This result confirmed that the chromosomal insertion of the tetra-$(His)_6$-tag on the

C-termini of L12 proteins did not have any negative affect on the ribosomal function in translation factor-mediated peptide bond formation. The higher activity seen with affinity-purified ribosomes could be due to higher homogeneity of the JE28 70S ribosome and faster preparatory speed in the affinity method. The ribosome purified conventionally by ultracentrifugation method contained some free 50S and 30S subunits together with 70S as evidenced in sucrose gradient analysis (Figure 3b), which could be the reason for their lower activity.

Furthermore, affinity-purified JE28 ribosomes were subjected to a cell-free coupled transcription–translation system for the synthesis of the fluorescent protein mCherry (28) from its DNA template. The synthesis of the active mCherry protein was followed by the gain of its fluorescence with time at 610 nm (Excitation at 500 nm) (Figure 4c). For comparison, wild-type ribosomes purified in the conventional method were also subjected to the same assay. The yield of the protein with JE28 ribosomes (Figure 4c, inset) was little higher than that with MG1655 ribosomes. This result confirms that the tetra-$(His)_6$-tagged JE28 ribosomes are highly active in all steps of protein synthesis.

Use of affinity method for the isolation and re-association of the ribosomal subunits

The presence of the tetra-$(His)_6$-tag only on the 50S subunit, and not on the 30S subunit enabled us to develop an easy method for purification of ribosomal subunits using affinity chromatography. The subunits of JE28 ribosomes were separated by dialysis or dilution in low-Mg^{2+} buffer (Materials and methods section) and then loaded in the HisTrap^{TM}HP column. Figure 5a represents the elution profile of the column with two distinct peaks (i) in flow-through and (ii) in high imidazole concentration. The first peak (flow-through) when pooled and analyzed in sucrose gradient, showed only 30S subunits and the second peak eluted with 150 mM imidazole was identified as 50S subunits (Figure 5b). The subunits, thus isolated, were found functionally active in IF2-mediated subunit association assay (27) (data not shown). The 30S subunits could be successfully re-associated (85–95%) with the tetra-$(His)_6$-tagged 50S subunits retained on the column by incubation with high-Mg^{2+} buffer.

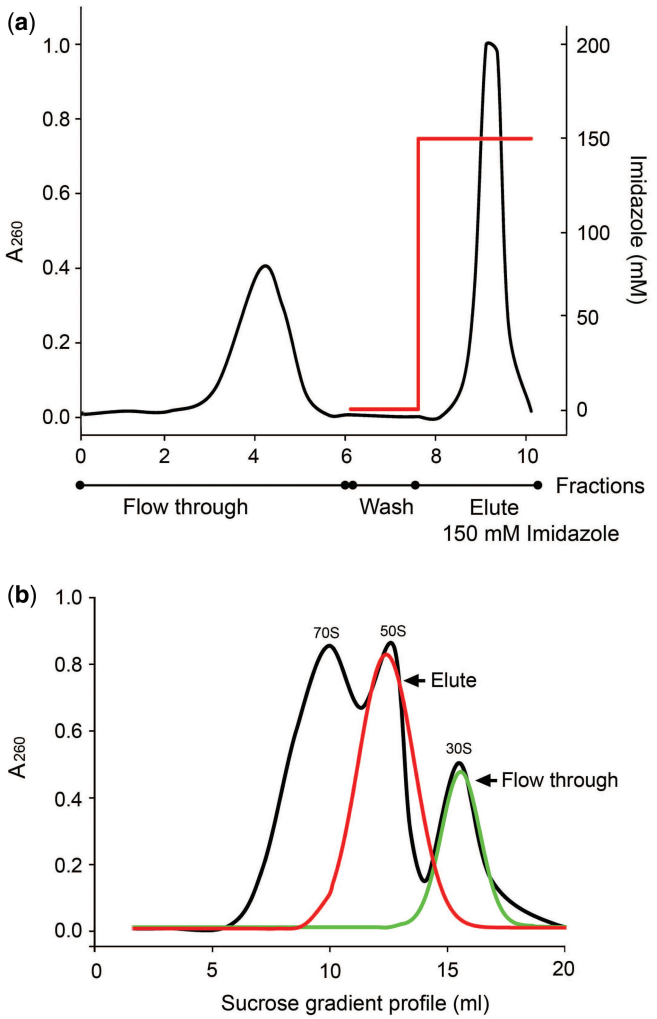

Figure 5. Isolation of the ribosomal subunits in affinity purification method. (**a**) Isolation of the ribosomal subunits from JE28 on HisTrap^{TM}HP Ni^{2+}–sepharose column. The JE28 ribosomes were incubated in low-Mg^{2+} buffer for subunit separation. The mixture was loaded on the Ni^{2+}–sepharose column in the same buffer and two peaks were eluted (1) in flow-through and (2) with 150 mM imidazole. (**b**) Sucrose density-gradient analysis of the peaks obtained above showed that the first peak eluted in flow through contained 30S subunits (green line) and the second peak eluted with imidazole contained 50S subunits (red line). A mixture of 70S, 50S and 30S ribosomes were run in the sucrose gradient in parallel as control (black line).

Scopes and applications of the affinity-tag-based purification method

The purification of His-tagged ribosome and its subunits from *E. coli* JE28 using affinity chromatography can be easily scaled up for high-throughput ribosome purification without significant changes in the infrastructure. This method is quite cost effective both in terms of time and reagents, compared to other methods of ribosome purification and therefore can be employed for commercial purposes as well. Since the His-tagged ribosomes from JE28 are highly active in translation they will be useful in analytical functional assays as well as in cell-free protein synthesis systems. A future goal is to develop a continuous-flow cell-free system where His-tagged 50S subunits from JE28 will be fixed on a solid matrix and 30S subunits with all other components will be recycled for continuous production of custom proteins and peptides.

The tetra-$(His)_6$-tagged ribosomes can be used to isolate functional translation complexes bound with translation factors for structural studies by cryo-EM or X-ray crystallography. It is also useful for the purification of *E. coli* ribosomes carrying mutations in essential rRNA or r-protein genes, which usually degrade fast and is therefore difficult to obtain in the conventional method. The affinity tag with the drug marker can be moved from JE28 to the mutant *E. coli* by generalized transduction with bacteriophage P1 (22) and then the mutant ribosomes can be purified by affinity chromatography.

Finally, we propose a general use of this affinity-tag-based purification method. In principle, it can be employed for purification of any macromolecular complex from *E. coli* and other bacteria. In the first step, the sequence encoding an affinity tag is to be inserted at the chromosomal location of a regular component of the complex, preferably well-exposed on the surface and present in multiple copies. Furthermore, this chromosomally fused tag can be used for isolating macromolecular complexes using the simple affinity purification method.

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