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Isolation of bacterial compartments to track movement of protein synthesis factors

Hanchao Zhao [1], Susan A. Martinis *

Department of Biochemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Ave., Urbana, IL 61801, USA

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ABSTRACT

Aminoacyl-tRNA synthetases (AARSs) comprise an enzyme family that generates and maintains pools of aminoacylated tRNAs, which serve as essential substrates for protein synthesis. Many protein synthesis factors, including tRNA and AARSs also have non-canonical functions. Particularly in mammalian cells, alternate functions of AARSs have been associated with re-distribution in the cell to sites that are removed from translation. Sub-fractionation methods for *E. coli* were designed and optimized to carefully investigate re-localization of bacterial AARSs and tRNA that might aid in conferring alternate activities. Cell fractionation included isolation of the cytoplasm, periplasm, membrane, outer membrane vesicles, and extracellular media. Specific endogenous proteins and RNAs were probed respectively within each fraction via Western blots using antibodies and by Northern blots with primers to unique regions of the nucleic acid.

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1. Introduction

Bacterial cells are organized with membrane barriers separating the cytoplasm and periplasm into two compartments. In the case of gram-negative bacteria such as *Escherichia coli*, a thin peptidoglycan layer is sandwiched between an inner and outer membrane that also defines the periplasmic space. The lipo-polysaccharide-containing outer membrane houses protein machinery to exchange small molecules such as nutrients and waste products with the exterior of the cell. Under certain conditions, outer membrane vesicles (OMVs) that host macromolecules also bleb off of bacterial cells [1].

Proteomic analysis of OMVs that were released from *E. coli* revealed that many protein synthesis factors were enclosed in the membrane-bound compartment [2]. These included proteins from the ribosome such as ribosomal protein S3, as well as aminoacyl-tRNA synthetases (AARSs), for example, alanyl- (AlaRS), glycyl- and phenylalanyl-tRNA synthetases. In addition, RNA-derived fragments from tRNA, 4.5S RNA and tmRNA were found in the OMVs [3]. The biological function of OMVs and the purpose of their contents remain to be discovered. However, these results did support that critical macromolecular protein synthesis factors from the cytoplasm, must be transported to the bacterial cell's periplasmic space and/or its outer membrane to be engulfed by the OMV upon its formation and release.

The family of AARSs is responsible for the first step of protein synthesis in the cytoplasm, where they synthesize and provide accurately charged tRNAs to the ribosome for translation. Each AARS typically represents just one of the twenty standard amino acids to catalyze aminoacylation of its cognate tRNA with the correct amino acid. In eukaryotic cells, charged tRNAs, as well as AARSs and other protein synthesis components have also been found in the nucleus [4] suggesting that translation might occur outside of the cytoplasm.

In addition to its housekeeping functions in protein translation, AARSs have also acquired alternate functions that are separate from aminoacylation [5]. These often include re-localization from the cytoplasm. For example, secreted tyrosyl- [6], tryptophanyl- [7], and threonyl (ThrRS)- tRNA synthetases [8] function extracellularly via interactions with cell receptors that induce different specific responses such as inflammation, migration and cell-cell contacts. Seryl-tRNA synthetase contains a nuclear localization signal (NLS) that re-directs a fraction of the protein to block c-Myc binding to the *VEGFA* promoter. This suppresses VEGFA expression, thus impacting downstream angiogenesis and vascular development [9,10].

Examples of alternate function for bacterial AARSs include two separate cases that regulate their own expression. In an alanine-dependent manner, *E. coli* AlaRS binds specifically to a palindromic

* Corresponding author.
E-mail address: martinis@illinois.edu (S.A. Martinis).
[1] Present address: The Scripps Research Institute, 10550 North Torrey Pines Road, BCC379, La Jolla, CA 92037, USA.

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sequence that flanks the *alaS* gene's promotor site to repress *alaS* gene transcription [11]. The production of *E. coli* ThrRS is autoregulated in a mechanism where ThrRS competes with ribosome that binds to $tRNA^{Thr}$-like moieties in its own mRNA's operator region to repress translation [12].

Because protein synthesis factors were identified in *E. coli* OMV's, we hypothesized that bacterial AARS and tRNA molecules might be re-localized from the cytoplasm for protein synthesis or alternate functions. In some cyanobacterial species, a membrane-binding CAAD domain (cyanobacterial aminoacyl-tRNA synthetases appended domain) in glutamyl-, isoleucyl-, valyl- and leucyl-tRNA synthetases (LeuRS) anchors these housekeeping proteins to the thylakoid membrane [13]. *E. coli* LeuRS was also reported to interact with the cytoplasmic membrane under certain conditions that depended upon leucine availability and antibiotic stress [14]. To explore the relocalization path of AARSs that were identified in *E. coli* OMVs, we developed detailed fractionation protocols to probe the membrane, periplasmic space, cytoplasm, OMVs, and also extracellular media.

2. Materials

Cyanogen bromide (CNBr)-activated-Sepharose 4B resin (No. C9142) and Optiprep (60%; D1556) density gradient medium were purchased from Sigma-Aldrich. *E. coli* strains MG1655 [15] and SX1025 [16] were obtained from Coli Genetic Stock Center (CGSC) at Yale University (New Haven, CT). Synthetic DNA was purchased from IDT (Coralville, IA). *E. coli* strain DY330 (parent strain of SX1025 [17]) was a gift from Dr. A. Emili (University of Toronto). Polyclonal antibodies against human cytoplasmic AlaRS (PA5-29496) and *E. coli* OmpA (No.111120) were respectively purchased from Pierce Antibiotics and Antibody Research Corp. Antibodies for *E. coli* ProRS were a gift from Dr. K. Musier-Forsyth (Ohio State University). Antibody against *E. coli* ribosomal protein S3 (No. 373C9C3A1) was obtained from the Developmental Studies Hybridoma Bank, at the University of Iowa.

3. Isolation of OMV's and recovery of cell-free media

Proteomics previously identified mass spectrometry fragments of certain protein synthesis factors in OMVs that are released from *E. coli* [2,3]. Although the biological function of OMVs is unknown, this suggests that the subset of translation machinery could be dispatched to facilitate protein synthesis elsewhere. It is also possible that these components have been re-tooled for an alternate function that is external to the cell. As a first step in exploring these roles, we sought to isolate OMVs (Fig. 1) and utilize Western Blots to directly probe two proteins from the translation machinery: ribosomal S3 and AlaRS [2].

E. coli MG1655 cells were inoculated in 2 ml LB media and grown at 37 °C overnight. Cells from 1 ml of the culture were recovered by centrifugation at 5000*g* for 10 min, washed twice with 1 ml fresh LB media, and re-suspended in 100 ml fresh LB media. During early stationary phase, when the OD_{600} measured between 1.8 and 2.0, 50 ml of the culture were collected by centrifugation at 5000*g* for 10 min. The cell-free supernatant was concentrated 10-fold using Ultracel 3 K cut-off tubes (Amicon). The concentrated media was then filtered through a 0.2 μm membrane (Corning, 431220) to remove any remaining whole cell contaminants. OMVs with an average diameter between 20 and 200 nm pass through the filter with the filtered media.

The filtered media fraction was centrifuged at 150,000*g* (or 40,000 rpm in a Beckman SW 60 Ti rotor) for 3 h to pellet OMVs. The pellet containing the OMVs was re-suspended in 200 μl sterile 1X PBS or alternatively, in 0.5 ml 50% Optiprep/1X PBS buffer. In the latter case, the sample was applied to the bottom of a step-density gradient that was prepared by gently stacking 0.5 ml each of 45%, 40%, 35%, 30%, 25%, and 20% Optiprep/PBS in that order (Fig. 2). A final layer of 0.7 ml 10% Optiprep/PBS was carefully added to the top. After ultracentrifugation at 200,000*g* (or 45,000 rpm in a Beckman SW 60 Ti rotor) for 20 h at 4 °C, 12 fractions of equal volume were collected from the top of the centrifuge tube [20].

The outer membrane protein OmpA would be expected to be localized in the OMVs, because these extracellular vesicles are comprised in part of the *E. coli* outer membrane. Western blot analysis that probed OmpA showed that due to their phospholipid content, OMVs migrated to the lower density region of approximately 35% Optiprep (Fig. 2). As first reported via proteomic profiling [2], our results using Western blots and antibodies against *E. coli* S3 supported that isolated OMVs contain the ribosomal protein. In addition, we probed the OMVs for AlaRS, which was also suggested to be exported in the OMVs [2]. However, Western blots using commercial antibodies failed to detect AlaRS. Since these antibodies were generated against human AlaRS, it is possible that their cross-reaction to *E. coli* AlaRS is not strong enough to detect low amounts.

4. Sub-fractionation of *E. coli* cytoplasm, membrane, and periplasm

Because ribosomal S3 was identified in OMVs that are released from *E. coli*, carrying parts of the outer membrane and periplasm, we investigated the re-localization path of ribosomal S3 from the cytoplasm. Specifically, we sub-fractionated *E. coli* to recover its cytoplasm where S3 and other translational machinery would be expected, as well as separately isolated the periplasm and total membrane (Fig. 1).

E. coli was grown in either rich LB (BP1426, Fisher Scientific) or M9 minimal media [14]. A single colony of *E. coli* strain MG1655 [15] was transferred to 5 ml media and incubated at 37 °C overnight. The overnight culture was then diluted 100-fold in the same media and grown at 37 °C. At specific OD_{600} values ranging from 0.5 to 2.0, *E. coli* cells were harvested by centrifuging at 5000*g* (or 6025 rpm in a Beckman JA17 rotor) for 10 min.

Sub-fractionation of *E. coli* to separate and recover periplasm, cytoplasm and the total membrane was carried out according to previously published protocols [19] with the following significant adaptations (Fig. 1). About 0.35 g of *E. coli* cells were incubated in 10 ml ice-cold buffer A [30 mM tris-(hydroxymethyl)aminome thane (Tris), 20% sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] for 5 min. The cells were spun at 10,000 g (or 8520 rpm in Beckman JA-17 rotor) for 10 min and re-suspended in 5 ml ice-cold 5 mM $MgCl_2$ to induce osmotic shock. After incubation on ice for 10 min with intermittent vortexing, spheroplasts (*E. coli* cells without periplasm and most of the outer membrane) were pelleted at 10,000*g* for 10 min. Spheroplasts were washed with 5 mM $MgCl_2$, spun again at 10,000*g* for 10 min, and stored at −80 °C.

The supernatant (4.6 ml) containing the periplasmic space fraction was combined with 92 μl of 1 M Tris, pH 7.5 to adjust the pH and then supplemented with 10 μl protease inhibitor cocktail (Sigma, P8465) that contained 23 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 100 mM EDTA, 2 mM Bestatin, 0.3 mM Pepstatin A and 0.3 mM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64). The periplasm was then purified from the supernatant by ultracentrifugation at 200,000*g* (or 45,000 rpm in Beckman SW 60 Ti rotor) for 2 h to remove outer membrane debris [18,21]. The periplasm fraction was aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C before use.

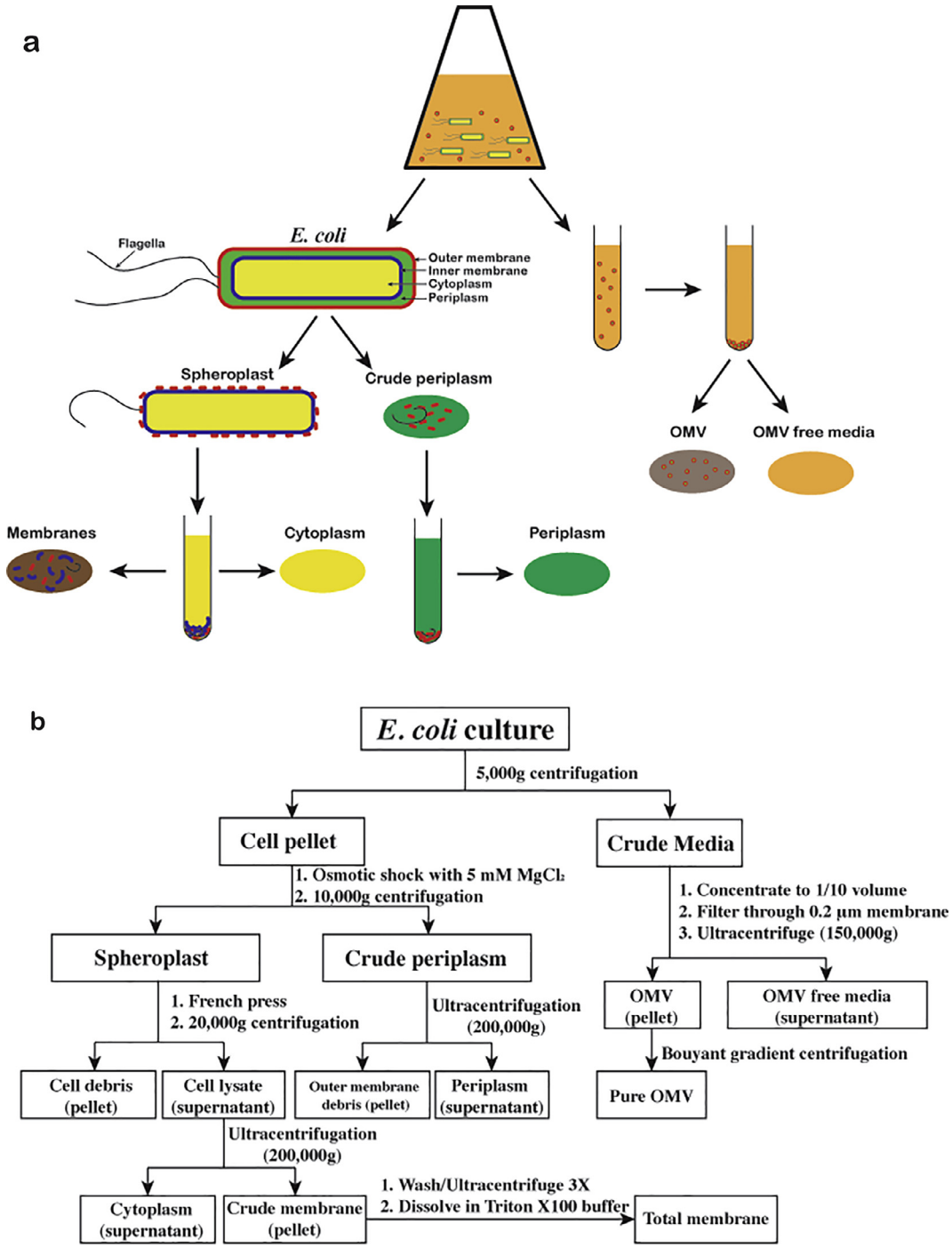

Fig. 1. Optimized methods of *E. coli* sub-fractionation and media analysis. a. Schematic cartoon of *E. coli* morphology and compartments. Cytoplasm, periplasm, inner membrane and outer membrane are colored in yellow, green, blue, and red, respectively. b. Flowchart of *E. coli* sub-fractionation and media analysis. Published protocols [18–20] were adapted for optimal sub-fractionation of *E. coli* and media analysis. Outer membrane vesicle is abbreviated as OMV. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Frozen spheroplasts were thawed on ice and re-suspended in 4 ml 1X PBS, as well as supplemented with 100 µl protease inhibitor cocktail, 2 µl 1 M dithiothreitol (DTT) and 10 µl phosphatase inhibitor cocktail (Santa Cruz Biotech, sc-45044) that contained 2.5 mM *p*-bromotetramisole oxalate, 500 µM cantharidin, and 500 nM microsystin-LR. The spheroplasts were homogenized three times by French Press (Thermo Spectronic, FA-078) at 1500 psi, followed by centrifugation at 20,000*g* (or 12,049 rpm in Beckman JA-17 rotor) for 30 min to pellet cell debris. The supernatant was subjected to ultracentrifugation at 200,000*g* for 4 h at 4 °C to separate cytoplasm from the total membrane [19]. The cytoplasm fraction was directly recovered from the supernatant and flash-frozen for storage as described above.

The total-membrane containing pellet was washed three times with 4 ml 1X PBS, followed by ultracentrifugation at 200,000*g* for 30 min. The washed pellet was re-suspended in 4 ml 1X PBS, which was supplemented with 2% TritonX-100 and 10 mM $MgCl_2$ [22,23] to dissolve membrane-derived phospholipids and membrane-bound proteins. After at least 2 h of incubation, the solution was ultracentrifuged at 200,000*g* for 1 h and the supernatant contain-

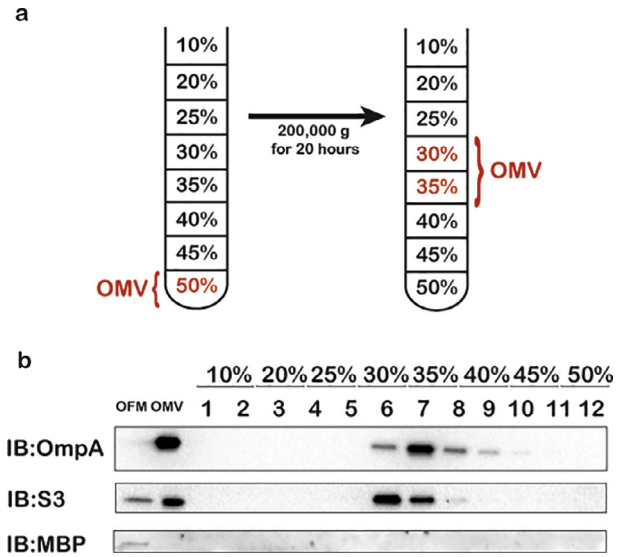

Fig. 2. Isolated *E. coli* OMVs contain OmpA and ribosomal protein S3. a. Scheme of buoyant ultracentrifugation to isolate OMVs. b. Buoyant ultracentrifugation fractions were analyzed by Western blot. OMV-free media (OFM; concentrated by 10-fold), crude OMV (OMV; concentrated by 25-fold) and twelve fractions of buoyant ultracentrifugation to purify OMVs were separated by SDS-PAGE and analyzed by Western blot using antibodies against OmpA and ribosomal protein S3.

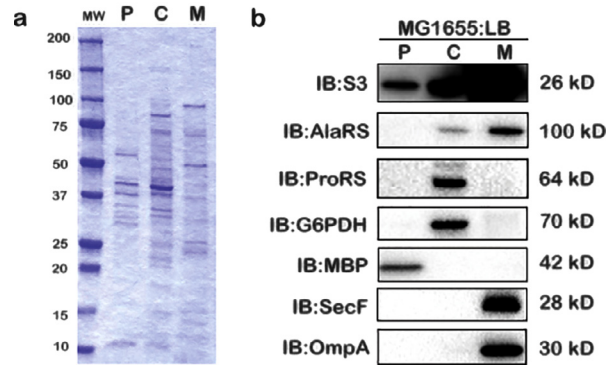

Fig. 3. Endogenous *E. coli* S3 and AlaRS proteins have diverse distributions in the cell. a. *E. coli* strain MG1655 was harvested at $OD_{600} \sim 0.5$. Sub-fractionated *E. coli* displayed distinct protein spectra for the isolated periplasmic space (P), cytoplasm (C) and membrane (M) fractions. b. Subfractions (~1 μg) were analyzed by Western blot using different antibodies. Antibodies against G6PDH, MBP, SecF and OmpA detect their target protein respectively in the cytoplasm, periplasmic space, inner and outer membrane.

ing the combined outer and inner membrane was recovered for flash-freezing, followed by storage at −80 °C.

The concentration of total protein for each fraction was determined using the Biorad DC protein assay (500–0116). Gradient dilutions ranging from 1- to 8-fold were incubated with the DC protein assay reagents, followed by absorption measurements at 750 nm. Each protein concentration was calculated based on standard curves that linearly correlated the 750 nm absorption measurements of BSA concentrations ranging from 50 to 1500 ng/μl.

Proteins from the isolated periplasm, cytoplasm and membrane fractions were separated via electrophoresis on a 4–20% polyacrylamide gradient gel (Biorad, 456–1096). Coomassie staining showed distinct banding patterns for each isolated fraction supporting that the *E. coli* compartments were cleanly separated (Fig. 3). In addition, Western blot analysis using antibodies against maltose binding protein (MBP), glucose 6-phosphate dehydrogenase (G6PDH), SecF, and OmpA confirmed complete separation of the respective *E. coli* periplasm, cytoplasm and membrane fractions (Fig. 3b).

Interestingly and in contrast to the other proteins probed, the ribosomal S3 protein was found in each of the fractionated *E. coli* compartments, as well as the OMV suggesting that it is ubiquitous throughout the cell (Fig. 3b). Based on its ratio to other proteins in each compartment, S3 was most abundant in the membrane fraction (~70% of total S3 protein). Regardless, its presence in both the periplasm and membrane fractions is consistent with its association with OMVs when it is released from *E. coli*.

Surprisingly, the human antibodies that failed to detect AlaRS in the OMVs (*vide supra*), showed that AlaRS is localized to the membrane fraction, as well as the cytoplasm where translation occurs. Indeed, relative to ProRS that is isolated from the cytoplasm as expected, only 60% of *E. coli* AlaRS is localized in the cytoplasm. Normalization and extrapolation to the volume of sample that was recovered during the sub-fraction revealed that about 40% of the cell's AlaRS population resides in the membrane fraction.

The C-terminus of AlaRS has a high pI of 9, which could anchor the protein to the negatively charged membrane. It is possible that AlaRS is simply localized to the inner membrane to perform its aminoacylation role in protein synthesis, where it would be associated with membrane-bound ribosomes. Alternatively, AlaRS could perform a non-canonical function when it is localized to the membrane. This might involve its amino acid-dependent role of AlaRS role in regulating the transcription of its gene [11]. Based in general on the massive expansion of functional roles for tRNA synthetases in eukaryotes [5], AlaRS or perhaps a modified AlaRS could also be an important partner in cell signaling.

5. Determination of RNAs in the periplasmic space, cytoplasm and total membrane

Because AlaRS was found predominantly in the membrane, we sought to determine if $tRNA^{Ala}$ was also co-localized to the membrane. Interestingly, RNA-Seq analysis indicated that $tRNA^{Ala}$ or at least a fragment of the tRNA was re-localized to *E. coli* OMVs [3]. We hypothesized then that $tRNA^{Ala}$ association with the membrane, possibly via AlaRS, could provide a mechanism for the RNA's inclusion into the OMV.

We utilized the fractionated *E. coli* cells that were analyzed above for protein and probed for RNA in not only the cytoplasm and membrane fractions, but also the periplasmic space and cell-free media that we had recovered from different growth phases. Cell-free growth media was concentrated to 10-fold and filter-sterilized. To recover RNA, each subcellular fraction of *E. coli* or concentrated media, was adjusted to contain final concentrations of 300 mM sodium acetate, 10 mM EDTA at pH 5.2. Protein was removed by extraction with acidified phenol/chloroform/isoamyl alcohol mixture (125:24:1, pH 4.3), followed by ethanol precipitation. The RNA-containing pellet was washed twice in 70% ethanol, vacuum-dried and dissolved in RNase-free water. Concentrations of the total RNA were determined based on absorption at 260 nm using a Nanodrop 2000 spectrophotometer (Thermo, Waltham, MA).

The RNA in each periplasm, membrane and cytoplasm fraction was radio-labeled for analysis on polyacrylamide gels. Specifically, 5 μg of extracted total RNA were treated with 20 U calf intestinal alkaline phosphatase (CIP, NEB) at 37 °C for 1 h in water to remove its 5′-phosphate group. The CIP-treated RNA was phenol-chloroform [phenol/chloroform/isoamyl alcohol mixture (125:24:1, pH 4.3)] extracted, followed by ethanol-precipitation at −20 °C overnight. The precipitated RNA pellet was washed twice with 70% ethanol, vacuum-dried and re-suspended in RNase-free water. Concentrations of recovered RNAs were determined via optical absorbance at 260 nm. An aliquot of 500 ng from each

sample was labeled with γ-[^{32}P]-ATP (6000 Ci/mmol, BLU002-Z250UC, PerkinElmer) using 10 U T4 polynucleotide kinase (NEB, Catalog # M0201S) at 37 °C for 1 h [24]. The radiolabeled RNA that was isolated from the *E. coli* periplasm, cytoplasm, and membrane were separated by electrophoresis using a 15% acrylamide-TBE gel containing 7 M urea (Fig. 4).

As would be expected, RNAs isolated from the cytoplasm exhibited a spectrum of bands that were representative of tRNA (75–90 base pairs), 5S (120 base pairs), tmRNA and 23S/16S RNA (>150 base pairs). Membrane-localized RNAs had a wide spectrum of sizes ranging from 10 to 150 bases. We hypothesized that these RNAs largely reflected mRNAs that co-localized with membrane-bound ribosome. In addition, discrete bands in the cytoplasm were identified at about 30 and 40 base pairs. It is possible that major bands of less than 50 base pairs are involved in RNA interference mechanisms or even tRNA fragments that are eventually found in the media [3]. Alternatively, degradation *in vivo* or during the extraction process could have resulted in lower molecular weight bands. In contrast, the majority of RNA lengths in the periplasmic space were less than 40 bases.

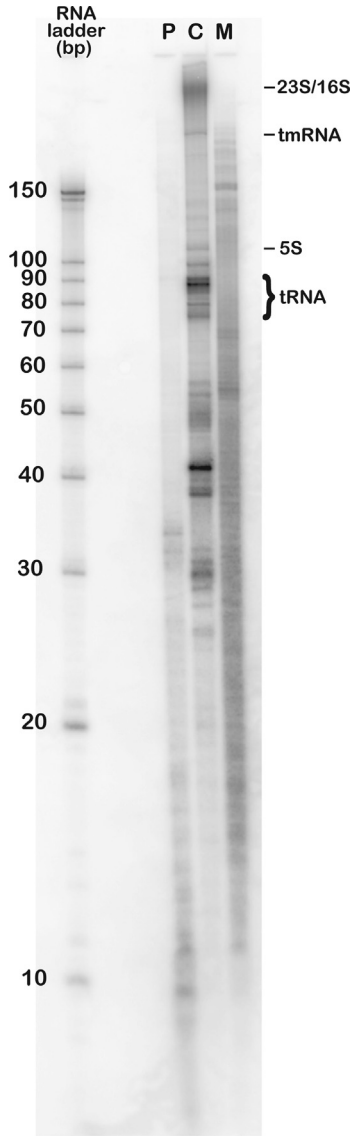

Fig. 4. Isolation of RNA from *E. coli* sub-fractionated samples. About 60 ng of radiolabeled RNA that were isolated from the *E. coli* periplasmic space (P), cytoplasm (C) and membrane (M) were separated by electrophoresis. A Decade RNA marker (left side, Ambion, AM7778) was used as standard.

6. tRNA recovery and detection

Although tRNA had been primarily detected in the cytoplasm via radiolabeling, we hypothesized that small amounts of full-length tRNA or its fragments could be co-localized with OMVs beneath the threshold of detection. Therefore, we designed radiolabeled DNA probes to detect specific tRNA isoacceptors. The relative binding affinity of each probe was calculated based on Northern blot results to normalize the measured extracellular tRNA levels for comparison.

The DNA probes were designed to specifically hybridize to $tRNA^{Ala}_{UGC}$, as well as controls that included $tRNA^{Pro}_{CGC}$ and $tRNA^{Ile}_{GAU}$ to assess and measure the levels of different tRNA isoacceptors (Fig. 5). Each probe of 31 or 32 nucleotides reversely complemented the 5′-half of the respective tRNA molecule (Table 1). About 40 pmol of each nucleic acid was [^{32}P]-end-labeled using 40 U T4 polynucleotide kinase at 37 °C for 1 h as described above. The reactions mixture was purified from unincorporated γ-[^{32}P]-ATPs using Chroma spin TE30 columns.

An aliquot of 5 μg extracted RNA was separated by electrophoresis on a 15% acrylamide-TBE gel containing 7 M urea. Electrophoresis was carried out in 1X TBE buffer at 300 V for about 1 h, until the bromophenol blue dye in the loading buffer migrated to the bottom of the length of gel. The RNAs were transferred to a nylon membrane (Hybond-N, Cat#RPN303N, GE Healthcare) in ice-cold 1X TBE at 80 V (400 mA) for 2 h, and then UV-cross-linked in a UV Stratalinker 1800 (Stratagene). In a 42 °C hybridization oven, the cross-linked nylon membrane was incubated in 10 ml pre-hybridization buffer (NorthernMax Prehyb/hyb buffer, Cat#AM8677, Ambion) for 2 h with gentle rotation. The nylon membrane was placed in a 50 ml Falcon tube with the side containing the RNA, facing the interior. Radiolabeled DNA probe was heated at 95 °C for 1 min and put on ice for 10 min before adding to the pre-hybridization buffer. The nylon membrane was incubated with rotation in the Falcon tube at 42 °C overnight.

On the next day, the nylon membrane was washed once with 50 ml 2X saline-sodium citrate (SSC; diluted from Ultrapure 20X SSC, Cat#15557–044, Invitrogen), 0.1% SDS buffer in a flask for 15 min with rotation. Two additional washes were carried out with 50 ml 0.1X SSC, 0.1% SDS for 15 min each at room temperature. The wet membrane was covered with parafilm and exposed to the storage phosphor screen (Fuji film, BAS cassette2 2040) overnight. The exposed screen was scanned using a Storm 840 phosphoimager (Amersham Pharmacia Biotech). The density of each spot was quantified using ImageQuant 5.2 software (GE Healthcare).

7. Determination of relative DNA probe binding affinities

The relative *in vivo* abundance of tRNA isoacceptors in the cytoplasm could dictate variations in concentrations measured by Northern blots, particularly those that are found in non-canonical sites outside the cytoplasm. Previously, Dong et al. [25] determined the *in vivo* concentration of each tRNA isoacceptor in fast-growing *E. coli* (2.5 doublings per hour), at approximately early log phase similar to the time frame that we collected *E. coli* cells to isolate RNA. We compared Northern blot densities for tRNA isolated from the cytoplasm to the relative abundance of tRNA reported previously using Northern blots, but with shorter probes [25].

To quantitate relative concentrations of tRNA isoacceptors, we measured the Northern blot band densities that reflected a particular tRNA isoacceptor when 2 μg cytoplasmic crude RNA (Fig. 6a, lane 4) were loaded. The bands for $tRNA^{Ile}$ and $tRNA^{Ala}$ were quantified and normalized to $tRNA^{Pro}_{CGG}$ as shown in Table 2. As expected, the band densities for $tRNA^{Ala}$ and $tRNA^{Ile}$ were significantly higher than the density for $tRNA^{Pro}$. The copy number for $tRNA^{Pro}$ is only

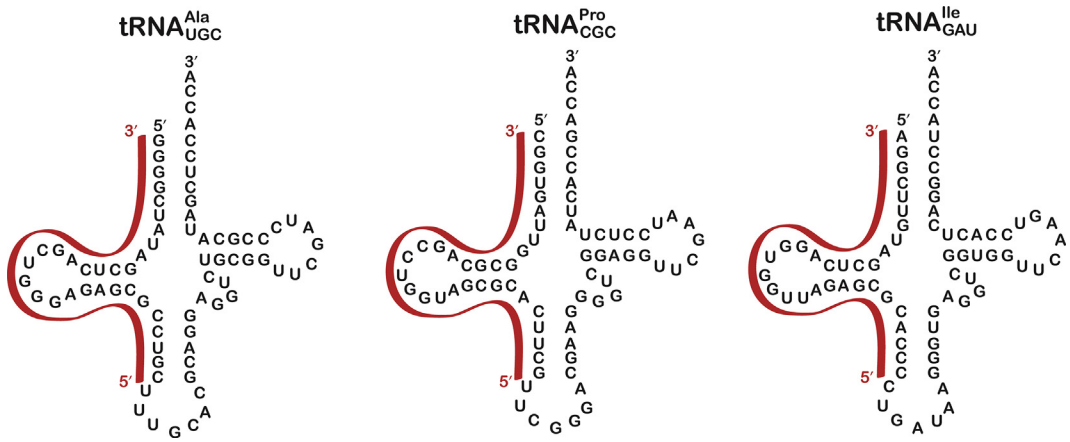

Fig. 5. Design of DNA probes to selectively identify *E. coli* tRNA isoacceptors. Synthesized DNA probes anneal to the tRNAs from the 5′-end to the penultimate residue prior to the sequence of the anticodon loop.

Table 1
Sequence and GC content of each Northern blot tRNA probe.

Target tRNA isoacceptor	Sequence (5′ to 3′)	GC content (%)	Length (bp)
tRNA$^{Ala}_{UGC}$	GGGGCUAUAGCUCAGC UGGGAGAGCGCCUGC	67.7	31
tRNA$^{Pro}_{CGG}$	CGGUGAUUGGCGCAGC CUGGUAGCGCACUUCG	65.6	32
tRNA$^{Ile}_{GAU}$	AGGCUUGUAGCUCAGG UGGUUAGAGCGCACCC	59.4	32

Table 2
Relative binding affinities of DNA probes that anneal to tRNA in Northern blot.

tRNA Type	tRNA^{Ala}	tRNA^{Pro}	tRNA^{Ile}
Anticodon	UGC	CGG	GAU
E. coli genome copy number	3	1	3
Northern blot Density (2 μg RNA in Cytoplasm)	13	1.0	6.7
in vivo Concentration[1] (μM)	21.0	2.67	24.7
Relative binding affinity[2]	1.7	1.0	0.72

[1] Ref. [25].
[2] "Relative binding affinity" is normalized to tRNA$^{Pro}_{CGC}$ data.

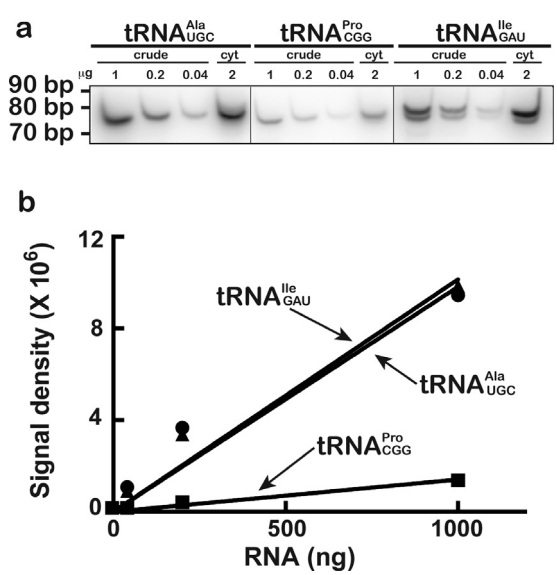

Fig. 6. Radiolabeled probe binding to target tRNAs. a. RNA from either crude *E. coli* tRNA (lane1, 1 μg; lane 2, 0.2 μg; lane 3, 0.04 μg) or the sub-fractionated *E. coli* cytoplasm (lane 4, 2 μg) was separated on a 15% denaturing gel. DNA probes designed to the 5′-half of specific tRNA isoacceptors were used for Northern blot analysis. b. Spot densities were quantified using ImageQuant 5.2 software (GE Healthcare). The increasing signal of each tRNA correlates with the absolute amount of tRNA inputted. The R square values of tRNA^{Ala}, tRNA^{Pro}, tRNA^{Ile} binding curves by linear regression fitting are 0.95, 0.99, 0.98 respectively.

one, while the other two tRNA isoacceptors have a higher copy number of three. This was also reflected by increased *in vivo* concentrations that were previously measured for tRNA^{Ala} and tRNA^{Ile}, relative to tRNA^{Pro} [25].

The relative abundance of the tRNA isoacceptors that were assessed by Northern blot analysis were further quantified by examining DNA probe binding affinities. Although each of the DNA probes were designed to have similar length and binding regions on the 5′ half of the tRNA, their sequence variation and the context of those variations could result in varied binding affinities to their target. We characterized the binding affinities by testing the DNA probes with increasing and known concentrations of tRNA target. Crude tRNA from *E. coli* (MRE600, No.10109550001, Roche) was separated by electrophoresis on a denaturing acrylamide gel. Analysis using increasing concentrations of tRNA on Northern blots determined that each of the three probes bound to their tRNA targets in a linear manner with high R values (Fig. 6).

The relative binding affinity (A) of each probe was calculated by dividing the quantified target tRNA density (D) by *in vivo* concentrations for tRNA that were obtained from the literature (C; Table 2):

$$\mathbf{A} = \mathbf{D}/C \tag{1}$$

The binding affinities of each of the three probes that we designed were comparable with up to a 1.7-fold difference. These relative binding affinities that were calculated based on tRNA isolated from the cytoplasm can be used to normalize the Northern blot signal of each tRNA isoacceptors that are isolated from other compartments of the cell, as well as the extracellular media.

8. Discussion

Sub-fractionation of *E. coli* to recover proteins from its different membrane, cytoplasmic, and periplasmic space compartments, as well as extracellular OMVs and cell-free media identified unexpected re-localization of certain proteins that are essential to the translation machinery. For example, the S3 ribosomal protein appeared to be delocalized throughout all the cell compartments. We also found S3 protein in the extracellular OMVs, as previously reported by proteomic analysis [2]. It is not clear why this

component of the ribosome's small subunit that interacts with mRNA in the translation initiation complex [26] was found so ubiquitously outside of the cytoplasm where protein synthesis occurs.

We hypothesize that the S3 protein carries out alternate activities in different compartments. Several alternate functions have already been identified. For example, extra-ribosomal activities of the S3 protein have been implicated in DNA repair [27], apoptosis [28] and host-pathogen interactions [29]. Unique alternate activities might stem from the S3 protein's housekeeping function that has been shown to enhance processivity by unwinding mRNA [30] for the ribosome. It is possible then that its RNA-binding capacity is used in other ways that might be idiosyncratic to the compartment that it is re-localized.

Although we could not detect AlaRS in the OMVs as previously predicted by proteomic analysis and mass spectrometry [2], we did discover that it is associated with the membrane of *E. coli* in relatively high abundance. *E. coli* AlaRS lacks a clear membrane-binding domain akin to some cyanobacteria AARSs that are embedded in the membrane. It is likely to be associated via protein-protein interactions with the membrane for either its role in protein synthesis or an alternate activity.

E. coli AlaRS remains the only AARS reported to repress its own transcription. It binds to the promoter region of the *alaS* gene. This AlaRS-DNA interaction is enhanced at increasing intracellular alanine concentrations [11]. Perhaps, *E. coli* AlaRS is recruited to the membrane in an alternate conformation that is triggered by binding mimetics of its house-keeping substrates.

Some eukaryotic AARSs relocalize to other non-translation based sites upon specific stimuli [6,9,31]. This re-localization is usually associated with a unique alternate function of the particular AARS or its association with other biomolecules within the target compartments. In contrast, prokaryotic cells are less compartmentalized and their AARSs have long been assumed to exist only in the cytoplasm, albeit in cyanobacteria, some AARSs are naturally anchored to the thylakoid membrane. These AARSs play critical roles under nitrogen-limiting conditions [13].

Precise sub-fractionation of the *E. coli* cell into separate compartments, identified that ribosomal protein S3 and AlaRS are distributed in high amounts in the membrane. This relocalization is an important first step to discovering new functions for housekeeping proteins in bacteria. Using *E. coli* as a model organism, our methods provide a comprehensive way to analyze the localization of AARSs and tRNAs at the sub-cellular scale. We anticipate that these methods to identify and characterize AARSs outside of the cytoplasm will reveal novel functions of bacterial housekeeping proteins and RNAs.

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