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Identifying *Escherichia coli* Factors that Selectively Bind the mRNA of Secreted Proteins

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Abstract

In this study we assay that the mRNA encoding secreted proteins; periplasmic protein propyl isomerase chaperone SurA and the phosphatase phoA would preferentially interact with several proteins rather than Ffh secretion factor. As well as the secreted proteins, the mRNAs encoding the cytosolic proteins cytoplasmic protein 3-isopropyl malate dehydrogenase (IsodH) and gMP reductase have the same interactions. We performed the pull-down assays using SurA mRNA as bait that showed that mostly interested proteins corresponding to the Staphylococcus aureus SRP protein and S. aureus FtsY-like Protein. Therefore, the Nucleoid-associated protein, YbaB and multifunctional conjugation protein TraI were recognized. Curiously, we were able to identified the following proteins; the fused protein chain initiation factor 2 (IF-2), DEAD/DEAH box helicase protein, multifunctional conjugation protein TraI, Acetyltransferase GNAT family protein I and 3-isopropylmalate dehydratase enzyme that interact with the cytosolic IsodH mRNA. Particularly, it was found that the least enzyme involved in functions associated with mRNA translation in the case of in E. coli and thus involved in the regulation of mRNA products. As well as 3-isopropylmalate dehydratase enzyme the ATP-dependent helicase Lhr also has a role in mRNA control. Beside the previous finding, in the case of the PhoA mRNA many proteins such as Poly (A) polymerase I enzyme (PAPI), 30S ribosomal protein S10, 50S ribosomal protein L28, Putative type II secretion system L-type protein YghE and Fumarate and nitrate reduction (FNR) regulatory protein were found. The key factor, Ffh, however was not amongst them.

Keywords: *Protein Secretion; mRNA Binding; 3-Isopropyl Malate Dehydrogenase; Alkaline Phosphatase Precursor; PhoA*

Introduction

Polypeptides that translocate across the inner membrane in bacteria typically contain N-terminal signal sequences (20 - 30-residue long) as part of pre-protein. These signal sequences are cleaved off from the pre-protein following membrane translocation to generate the mature protein [18,21,23,38,39]. These signal sequences play a decisive role in targeting the protein to the Sec machinery and direct the protein across the inner membrane in bacteria [23].

It is well recognized that the pre-proteins destined for export through the inner membrane are recognized by factors such as the ribonucleoprotein signal recognition particle (SRP). This particle is a complex consisting of a 4.5S RNA and a 54-kDa Ffh protein, a homologue of eukaryotic SRP [2,32]. The SRP targets the nascent amino acid chain/ribosome complex to the translocon within the plasma membrane. Once the SRP-pre-protein complex is bound to SRP receptors and the ribosome is docked on the SecYEG translocon, the polypeptides are translocated across the membrane [2,18,21,32,38]. It is well documented that the SRP targets the highly hydrophobic N-terminal signal sequences of nascent secretory and membrane proteins during co-translational translocation/insertion, whereas SecB targets these substrates post-translationally to the Sec translocase [23,38].

There have been several studies that show that changing the structure of the signal sequence can eliminate or reduce the efficiency of protein secretion [22,30,40,52]. However, other alterations in this sequence can enhance protein sorting or have no effect [33,39]. These observations may implicate other factors and pathways that participate in targeting these substrates to the membrane during mRNA translation.

It has been proposed that the mRNA that codes for a secreted protein can actively participate in protein targeting rather than or in addition to the N-terminal amino acid signal sequence [9,12,33,35,49]. This mRNA hypothesis was established following several studies on mRNA targeting and subcellular localization in eukaryotes and prokaryotes [9,33,35]. For example, it has been reported that fliC export by the flagellar system in *E. coli* requires the 5' untranslated (UTR) region [12].

In this study, the periplasmic protein propyl isomerase chaperone SurA, which is involved in the biogenesis of outer membrane proteins [4,48] was utilized to identify novel RBP and that may play a role in the secretory pathway in *E. coli*. This protein contains a cleavable signal sequence and was selected in this study as the test protein. The 3-isopropylmalate dehydratase, an enzyme involved in the biosynthesis of the amino acid leucine, was used as a control for the non-secreted protein [14,54]. Additionally, we examined the utility of the 5' UTR of the *E. coli* alkaline phosphatase PhoA (exported) and GMP reductase (cytoplasmic) mRNAs in the isolation of such cytoplasmic factors. Biotinylated mRNA transcripts of surA, phoA, isoH and gMP were generated using *in vitro* system. The transcripts were used as bait in pull-down assays with streptavidin-magnetic beads. Bound cytoplasmic factors were isolated by SDS-PAGE and subjected to identification using tryptic digestion and mass spectrometry. The technique used in this study did not identify any specific cytoplasmic factors with potential function in targeting the mRNAs tested. The validity of this experimental approach was confirmed indirectly by the isolation and identification of a number of RNA/DNA binding proteins. An unexpected result was the identification of 3-isopropylmalate dehydratase as an interaction partner with its mRNA bait.

Materials and Methods

Bacterial cultures

Escherichia coli strains B; ATCC 11303 and BL21 was cultured and maintained in LB (Fisher Scientific, Mississauga) liquid media. For solid media, agar was added to the liquid media at a final concentration of 1%. Long term storage at -80°C in LB media containing 10% glycerol was used to save the stock cultures. Liquid cultures were maintained at 37°C in a rotary shaker (Inova 4000, New Brunswick Scientific) at 200 rpm as a source of bacteria, bacterial DNA, and protein extracts.

Extraction of genomic DNA

Twenty ml from an overnight culture of *E. coli* at 37°C/200 rpm were transferred to 50 ml polypropylene tubes containing 1.5 ml TNE buffer (0.1M Tris-Cl, pH 8.0, 0.15M NaCl and 20 mM EDTA) and washed thoroughly by three cycles of spin/resuspend at 2000 xg for 5 minutes/cycle. Following a final spin, the cells were collected and resuspended in 2 ml ice-cold 70% ethanol and incubated on ice for 20 minutes, collected by centrifugation and resuspended in 4.8 ml of TEST/LR buffer [0.1M Tris-Cl, pH 8.0, 20 mM EDTA, 0.5M sucrose, 1% (v/v) TritonX-100, 24 mg of lysozyme (30 µl of lysozyme). Following an incubation step on ice for 1h with occasional shaking, the suspension was transferred to a water bath set at 68°C for 10 minutes. Subsequently, 50 µl of 10% SDS was added and incubation was continued for an additional 15 minutes. Finally, 87 µl of 5M NaCl and 69 µl of CTAB/NaCl solution (1% N-acetyl-N, N, N -trimethylammonium bromide in 0.73M NaCl) were added and the tube contents were mixed by inversion and rotation. The extraction was followed by incubating the tube at 68°C for 15 minutes and then at -20°C for 30 minutes. The DNA was extracted from the cell lysate by the addition of 1 vol chloroform: isoamyl alcohol (24:1, v/v), mixing for 5 minutes, followed by centrifugation at 10000 rpm for 10 minutes to separate the organic phase from the aqueous phase. Added drop by drop, 2 vol 100% ice-cold ethanol were added to precipitate the genomic DNA for 30 second. A final centrifugation at 12,000 xg for 15 minutes and washing in ice-cold 70% ethanol produced the purified DNA.

Transcription by T7 RNA polymerase

To generate the mRNA transcripts, DNA templates from *E. coli* strains BL21 and ATCC11303 (See table 1 below) were synthesized using the polymerase chain reaction (PCR) mediated by oligonucleotides primers that are 40 base pairs long including the 20 bp of the T7 promoter sequence. The mRNA transcripts were generated *in vitro* using the MEGAscript T7 transcription Kit (Ambion, Austin, TX). Briefly, 20 µl or 40 µl reactions were assembled using the supplied UTP, ATP, GTP and CTP ribonucleotides and 1 µg of template DNA from *E. coli* strains BL21 and ATCC 11303. The mixture was assembled on ice and the reactions were started by shifting the temperature to 37°C. The reactions were terminated after 3h. Additionally, DNase treatment was used to remove the DNA template by adding 1 µl TURBO DNase to the reaction once its completed. The RNAs were purified using the Rapid Bacteria RNA Isolation Kit (Bio Basic) and the RNA concentrations were determined spectrophotometrically using a Shimadzu UV-2401PC Spectrophotometer (Hitachi, Tokyo, Japan).

Gene	Primers	Temp.	Fragment size (bp)
isodH	Forward:5'TAATACGACTCACTATAGGGATGGCTAAGACGTTATACGAAAAA-3' Reverse: 3'TAGCGAAGGTTTTGCCCG-5'	51 - 52°C	178
phoA	Forward:5'TAATACGACTCACTATAGGGAAAAAGTTAATCTTTTCAACAGCTGTC3' Reverse: 3'GCCCGGTTTTCCAGAACAG-5'	59 - 60°C	200

Table 1: *Primers used for the PCR reactions.*

Biotin labeling of mRNA

The biotin label enables the RNA probe to be immobilized with streptavidin that is beneficial to study such RNA interactions. RNA 3' End Biotinylation Kit (Pierce) was used to attach a single biotinylated nucleotide to the 3' terminus an RNA strand. The reaction mixture was prepared by combining of 50 pmol of test RNA, 40 U RNase inhibitor, 1X of 10X RNA ligase reaction buffer, 1 nmol of biotinylated cytidine (Bis) phosphate, 40 U of T4 ligase, 15% of PEG and up to 30 µl nuclease free water. The reaction was then incubated for 3h, 6h and 16h at 16°C. In order to extract the RNA ligase 100 µL of chloroform: isoamyl alcohol was added and tubes were centrifuged for 2 - 3 minutes to separate the phases. 10 µl of 5M NaCl, 1 µl of glycogen and 300 µl of ice-cold ethanol were added to the aqueous phase. The samples were then centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant discarded and the pellet was washed with 300 µl ice-cold 70% ethanol and air-dried the pellet was resuspended in 20 µl dH_2O.

Chromogenic detection of biotinylated mRNA and evaluation of labeling efficiency

The biotin chromogenic detection kit (Fermentas) was used to detect the biotinylated RNA on polyvinylidene difluoride membrane (PVDF). 30 ml of Blocking/Washing Buffer was used [1 volume of 10X Blocking/Washing Buffer with 9 ml dH_2O] to wash the membrane for 5 minutes at RT on platform shaker. The membrane was further blocked in 30 ml of Blocking Solution [1% (w/v) 0.3g of Blocking Reagent in 1X Blocking/Washing Buffer] for 30 minutes and then the membrane was incubated for an additional 30 minutes in 20 ml of diluted Streptavidin-AP conjugate [dilute concentrated Streptavidin-AP conjugate 5000-fold in Blocking Solution]. With moderate shaking the membrane was washed in 60 ml blocking/washing buffer for 15 minutes and repeated once with fresh blocking/washing buffer. The solution was discarded and then the membrane was incubated with 20 ml of detection buffer for 10 minutes using BCIP/NBT as substrates. To determine the labeling efficiency, spectrophotometric measurement of labeled RNA was perform by quantifying the amount of biotin at 500 nm in the samples in order to estimate the biotin concentration that attached on bait in compared with the Biotinylated IRE RNA Control from the Pierce RNA 3' End Biotinylation Kit using Shimadzu UV Spectrophotometer (Hitachi, Tokyo, Japan).

Protein extraction and UV crosslinking

Cells from an overnight culture of *E. coli* at 37°C/200 rpm were harvested by centrifugation at 15,000 rpm for 10 minutes. The pellet was resuspended in 1.0 ml lysis buffer [50 mM Tris-HCl, pH 7.5 (6.05 g/l), 150 mM NaCl (8.76 g/l)] and Lysozyme at 0.2 mg/ml was added. The pellet was then sonicated in an ice bucket using 3 x 10 sec pulses and then 1.0 ml lysis buffer containing 0.1% Triton X-100 was added. The soluble proteins (supernatant) were prepared by centrifugation (15,000 xg for 10 minutes), which was then used for the pull-down assay. Protein concentrations throughout this work were quantified using the BCA kit (Pierce Scientific). *E. coli* extracts were incubated with biotinylated RNA species, and a number of factors that are able to bind the RNA were enriched. The putative interacting factors were physically cross-linked to biotinylated-mRNA by exposure to UV-C light at 254 nM/120,000 μJ/cm^2 for 5 minutes (Microprocessor-Controlled UV Cross linkers). Therefore, the mRNAs and the bound interactome can be efficiently captured using pull down assays.

Pull-down assay

An aliquot of magnetic beads (0.8 mg beads/1 mg of *E. coli* extract) (Dynabeads, Invitrogen) was washed with TBS buffer, 7.4. The beads were then blocked with 5% casein hydrolysate in TBS [50 mM Tris-Cl, pH 7.6; 150 mM NaCl] for 2h at 22°C (RT) with gentle rotation followed by 3 washing in TBS buffer. Approximately 40 - 50 μg of biotinylated mRNA was incubated with 1 mg of *E. coli* protein extract in binding buffer (3 ml of 1X TBS [50 mM Tris-Cl, pH 7.6; 150 mM NaCl], 0.05% TritonX-100) at 4°C for 1h. The mixture then was added with coated beads for an additional hour at 4°C. Samples were boiled for 10 minutes following the addition of 15 μl of 2X SDS sample buffer [2 mL Tris (1 M, pH 6.8), 4.6 mL glycerol (50%), 1.6 mL SDS (10%), 0.4 mL bromophenol blue (0.5%) and 0.4 mL β-mercaptoethanol].

Diagonal 2D SDS-PAGE

Native polyacrylamide gel electrophoresis was used in this assay to separates proteins based on both proteins charge and its size. These gels were made of a 12% separating gel (3.4 ml H_2O, 2.5 ml 1.5M Tris-HCl pH 8.8, 4 ml 30% acrylamide/0.8% bis-acrylamide (w/v), 60 μl 10% (w/v) ammonium persulfate (APS), and 7 μl tetramethylethylenediamine (TEMED)) and a 4% stacking gel (3.1 ml H_2O, 1.25 ml 0.5M Tris-HCl pH 6.8, 0.65 ml 30% acrylamide/0.8% bis-acrylamide (w/v), 31 μl 10% (w/v) APS and 5 μl TEMED). 1 mg of *E. coli* extract were prepared in a non-reducing non-denaturing 2X sample buffer (38% glycerol, 0.1M Tris-HCl pH 6.8, 0.02% bromophenol blue, 0.1M DTT) to final concentration of 1X and then loaded into the wells. The gels were ran at a constant voltage of 200V for 45 minutes in Mini -PROTEAN Tetra System (BIO-RAD) with 1x native running buffer (144g glycine, 1/10 dilution of 30g Tris-HCl in 1L dH_2O; pH 8.3). Lanes from native-PAGE gel are cut and the strips were incubated in SDS sample buffer for 15 minutes at 22°C (RT). Strips were then rotated through 90° and placed into SDS-PAGE gel that either included phoA bait or has no transcript as control [34]. The Pierce Prestained Protein Molecular Weight Marker was used as a protein standard for protein ranging in size from 20 - 120 KDa. The gel was stained with Coomassie brilliant blue stain.

Protein identification using matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry

For in-gel digestion and extraction, the protocol provided by the Advanced Protein Technology Centre (http://www.sickkids.ca/Research/APTC/Map-Spectrometry/Sample-Protocols/In-Gel-Tryptic-Digestion-Protocols/index.htmL) was used. Gel pieces having a single protein band from the SDS-PAGE gels were placed in micro centrifuge tubes and de-stained by washing with 50 μl of 50 mM Ammonium bicarbonate (AmBic) for 5 minutes at RT. The gel pieces were then shrunk with 50 μl of 50% acetonitrile/25 mM ammonium bicarbonate for 10 minutes followed with 30 μL of 10 mM DTT to reduce the gel for 30 minutes at 56°C. The reduced proteins were subjected to alkylation with 30 μL of 100 mM iodoacetamide for 15 minutes in the dark and the supernatant was discarded. The gel pieces were then submerged in 14 μl of 50 mM ammonium bicarbonate containing 15 ng of Trypsin and incubated on ice for 20 minutes. An additional 20 μl of 50 mM AmBic were added to the gel pieces and incubated at 37°C overnight. Following the overnight incubation the supernatant was collected and transferred to a new microfuge tube. The tryptic peptides that remained in the gel pieces were extracted by sequential

treatment with 20 µL of 5% formic acid and 100% acetonitrile, for 10 minutes each treatment. All the supernatant were pooled and dried down using a SpeedVac concentrator (Fisher Electron Corporation, Savant 120) before being sent to the Advanced Protein Technology Centre at the Hospital for Sick Children (Toronto, Canada).

Results

Transcription of amplicons

Since the RNA binding factors were the intended target, a high yield of a particular mRNA was produced by conventional *in vitro* transcription reactions in the presence of the T7 polymerase enzyme. By modifying typical transcription reaction condition the reaction yields a total of approximately 100 µg of RNA. Between 100 - 200 bp of surA mRNA (Figure 1 lane 3) and isodH mRNA (Figure 1 lane 4) were generated and samples were visualized in 1% agarose gel while lanes 1 and 2 were represent the surA and isodH gene fragment transcripts. The transcription reactions of phoA and gMP gene fragments with a predicted size between 100 - 200 bp were also generated.

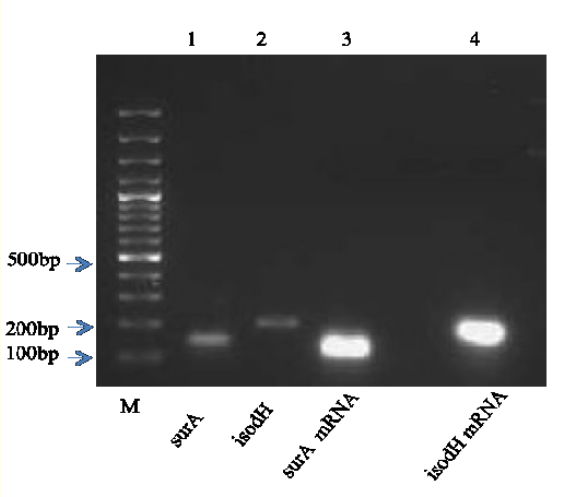

Figure 1: *Amplification of surA and isodH genes fragments. Agarose gel showing a PCR product of surA gene that were amplified at 57 °C in lane 1 and at 58 °C in lane 2 while in lane 3 an amplification of isodH gene fragment at 51 °C and at 52 °C were shown in lane 4. Bands shown between 100-200bp are the expected size of both genes fragments amplified reaction (predicted size 127 bp for surA and 178 bp for isodH). M= 100 bp plus DNA ladder (Fermentas).*

Quality of transcription product

Characterization of RNA quality was done using the Agilent 2100 Bioanalyzer. It is a simple and alternative method to determine the quantification and data analysis of RNA. After transcriptions of surA and isodH mRNAs from *E. coli* different concentrations of transcripts were indicated (Figure 2). The analysis determined that surA mRNA (23 ng/µl) and isodH mRNA (71 ng/µl) concentrations were enhanced after 4 h of incubation (lanes 1 and 2). However, the concentrations of surA mRNA (9 ng/µl) and isodH mRNA (3 ng/µl) decreased after 16h incubation (lanes 3 and 4).

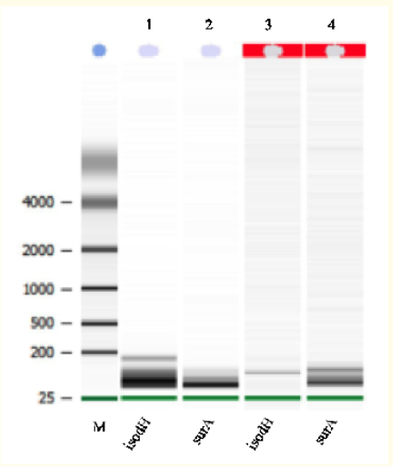

Figure 2: *Analysis of RNA transcription products using the Agilent Bio-analyzers. Bioanalyzer analysis was used to give a size estimate of the in vitro transcription reaction (using the surA and isodH gene fragments transcripts as template to generate RNAs) after incubation for 4 h (lanes 1 and 2) and for 16h (lanes 3 and 4). This method was also used to determine the quality of isodH and surA mRNAs. The following concentrations were determined after transcription for 4 h surA mRNA (23 ng/µl), isodH mRNA (71 ng/µl) while surA mRNA (9 ng/µl) and isodH mRNA (3 ng/µl) were determined after 16 hours of incubation. The gel indicates in a couple of mRNAs samples more than one size of RNA product in lanes 1 and 4.*

Estimation of labeling efficiency

Biotinylation of the transcripts in this study was performed not for detection but to be used as a target for the pull down assay. Addition of the biotin to the transcripts allowed the use of streptavidin-coated beads to specifically bind and pull down the transcripts along with any bound target protein. Results shown that 41% of surA, 36% isodH %, 43% of phoA and 38.5% gMP mRNAs were labeled at 37°C for 2h.

Detection of biotinylated RNA

Biotinylated surA and isodH transcripts were detected with streptavidin coupled to alkaline phosphates (AP) on PVDF membrane. Streptavidin -AP conjugates bind to the biotin - labeled mRNA and then visualized using chromogenic substrate for alkaline phosphatase BCIP/NBT, which produces a blue-purple precipitate (Figure 3).

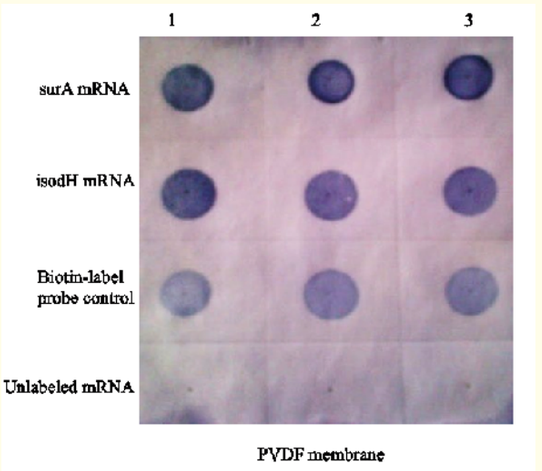

Figure 3: *Detection of biotinylated mRNA on PVDF membrane. The biotinylated reaction of surA and isodH mRNAs was performed for 3, 6 or 16 hours as shown in columns 1, 2 and 3 respectively. Biotin-label transcript control and unlabeled mRNA were detected using alkaline phosphatase-conjugated streptavidin from the biotin chromogenic detection kit.*

Pull-down assay

A pull-down assay employing biotinylated RNA to characterize and identify RNA binding proteins from *E. coli* was performed. To stabilize the nucleic acid protein interaction, UV crosslinking was used. Finally, the purified targeted proteins were visualized after electropho-

resis on an SDS-PAGE gel. The lanes of crude extract of *E. coli*, SurA and IsodH were identical and there was not a unique band generated from pull down assay. Another attempt at purifying RNA binding factors was performed. A duplicate amount of biotinylated RNA was cross linked to an *E. coli* proteins extract followed by an extra incubation at 4°C for 2h. Proteins were collected as before after binding the extract with Streptavidin-Coupled Dynabeads. Analysis of the proteins by electrophoresis on SDS-PAGE did not identify any target protein that specifically bound to surA mRNA or isodH mRNA although a number of proteins in the *E.coli* extracts were identified that bound non-specifically to the Dynabeads. The phoA and gMP transcripts were shown to interact with several protein species as indicated by the presence of specific bands following analysis on 15% SDS -PAGE of pull-down assays (Figure 4A). Additional experiments to isolate binding proteins in the *E. coli* extracts following an extra incubation with the a duplicate amount of same phoA and gMP transcripts, showed similar results with identification of similar protein bands as shown in figure 4B.

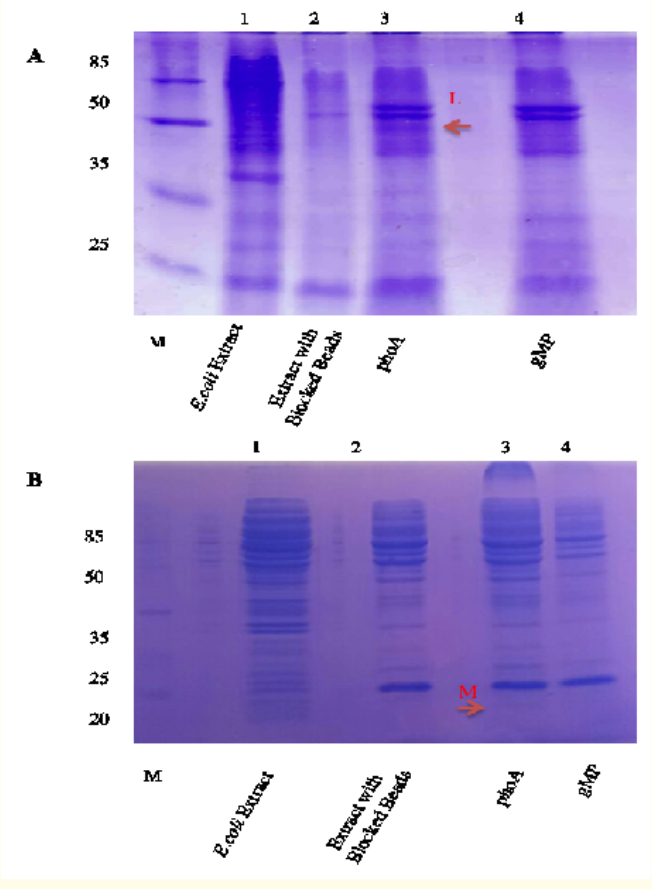

Figure 4: *Separation of proteins from the pull down assay using phoA and gMP transcripts as baits. The pull down assay with an extra incubation of transcripts with E. coli protein extract in binding buffer (3ml of 1x TBS [50 mM Tris-Cl, pH 7.6; 150 mM NaCl], 0.05% TritonX-100) is represented in panel B rather than panel A. Distinct bands marked (L) and (M) were subsequently subjected to trypsin digestion and identification using mass spectrometry .*

Diagonal 2D SDS-PAGE

After the separation of *E.coli* protein extract in native PAGE gels, each lane was cut and placed horizontally on denaturing gel with a 4% stacking and 12% resolving gel containing either *E.coli* extract as control (Figure 5A) or contain the transcript of phoA (Figure 5B).

The gels were stained with Coomassie brilliant blue and a diagonal migration of proteins was generated for both gels. An aggregation in figure 5B (spot N) refers to the binding of the mRNA to proteins in the extract. The binding of the mRNA to protein will change the effective migration of the protein away from the diagonal pattern seen in the extract alone. MALDI MS identification of spot (N) assigned it as Putative type II secretion system L-type protein YghE.

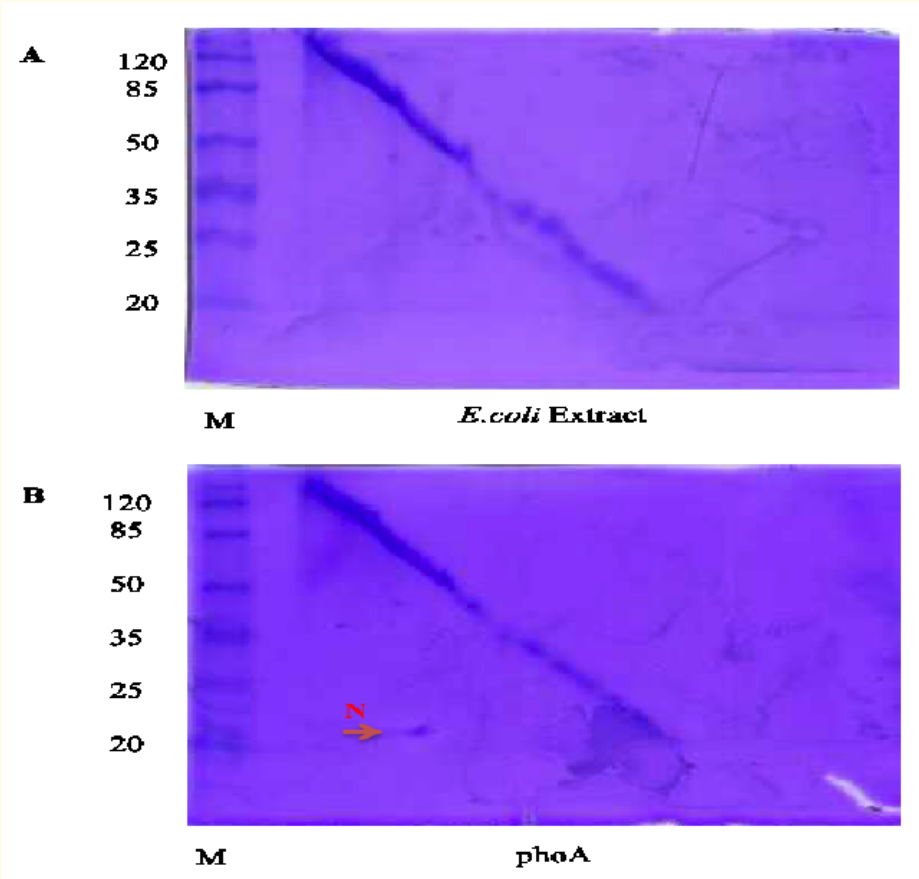

Figure 5: *RNA- protein interaction assay.*

After the 12% native polyacrylamide gels were performed with E. coli extract the cut lanes were pressed to 12% SDS-PAGE gel in order to capture any aggregation that might refers to RNA -protein complex. Electrophoreses of E. coli extract in gels contain either control (A) or contain the transcript of phoA generated from in vitro transcription reaction (B). A distinct spot marked (N) was subsequently subjected to trypsin digestion identification using mass spectrometry analysis. M=Marker (kDa).

Protein identification by mass spectrometry

Throughout all the RNA labeling and pull-down steps for the RNA binding factors there was one major band in the surA pull down assay consistently appeared at approximately 45 - 50 kDa. The band appeared on several of the gels and was tentatively putatively identified as a signal recognition particle (SRP) component, FtsY-like protein or DEAD/DEAH box helicase following MALDI-TOF-dependent characterization. In addition, at protein bands identified in the surA pull-down experiments between 20-40 kDa bands were putatively

identified as the Nucleoid-associated protein YbaB, and putative protein RhsE (or other possible proteins). As a final attempt to isolate the RNA binding factor, other protein bands present in the surA mRNA pull down assays were further excised for MALDI identification after a trypsin digestion step. The results of this analysis indicated that the proteins captured by surA pull downs included different candidates possibly identified as the HTH-type transcriptional regulator ZntR and the ABC transporter ATP-binding protein that might have several functions in *Escherichia coli*.

On the other hand, the MALDI mass spectroscopy results showed that the proteins that bound to the isodH mRNA included candidate proteins such as the Fused protein chain initiation factor 2 (IF-2), 3-isopropylmalate dehydrogenase and Transcriptional regulatory protein YehT. In additional pull-down attempts with the isodH mRNA, other proteins bands were identified using MALDI-MS as the DEAD/DEAH box helicase, Acetyltransferase, GNAT family and ATP-dependent helicase Lhr (Table 2).

Sample /*	Proteomics Analysis	Calculate Mass	Identify Protein	Protein sequence coverage	Score	Index
isodH-D 40-45*	MASCOT	-	Hypothetical Protein	30		gi|423037776
		39	3-isopropylmalate dehydrogenase	-	-	-
		97	Fused protein chain initiation factor 2 (IF-2)	-	-	-
		42	Protein KlaB	64	39	-
		50	ATP-dependent RNA helicase RhlE	55	31	-
	Protein Prospector	63	50S ribosomal protein L33	56.4	442	361087
	PROFOUND	100	ATP-dependent RNA helicase HrpA	27	-	gi|419936745
isodH-E 25-30*	MASCOT	19	Resolvase, N-terminal domain protein	34	-	gi|417246748
		27	Transcriptional regulatory protein YehT	57	32	-
		28	Fumarate and nitrate reduction regulatory protein	44	41	-
	Protein Prospector	-	N/A	-	-	-
	PROFOUND	-	N/A	-	-	-
isodH-F 15-20*	MASCOT	19	Resolvase domain-containing protein	44	-	gi|366161248
	Protein Prospector	-	N/A	-	-	-
	PROFOUND	-	N/A	-	-	-
isodH(G)/ 20-25*	MASCOT	16	Endopeptidase	34	-	gi|415778122
		17	bacteriophage lysis protein	45	-	gi|194430045
	Protein Prospector	-	N/A	-	-	-
	PROFOUND	-	N/A	-	-	-
isodH(H)/ 15-20*	MASCOT	16	DEAD/DEAH box helicase	13	-	gi|301026837
		16	ATP-dependent helicase Lhr	14	-	gi|15802067
		18	Acetyltransferase, GNAT family	-	-	-
isodH(K)/ 40-45*		191	Multifunctional conjugation protein TraI	41	48	

Table 2: *Proteins identified from pull down assay of IsodH mRNA using MALDI-MS identification.*

For the isolation of a specific protein factor that binds the phoA mRNA rather than gMP mRNA the pull down assay showed a unique band following SDS-PAGE for the phoA mRNA in figure 4A that was not present in extracts interacted with gMP mRNA in figure 4B or in the total *E. coli* extract. The MALDI mass spectroscopy identified the band (L) as the Poly (A) polymerase I at 50 kDa. The smaller 20 kDa band (band M) was similarly identified as the 30S ribosomal protein S10, the 50S ribosomal protein L28 and the fumarate and nitrate reduction regulatory protein in the second phoA mRNA pull down. Furthermore, beside all previous identification, the digestion of spot (N) in figure 5B was identified as Putative type II secretion system L-type protein YghE (See table 3).

Sample /*	Proteomics Analysis	Calculate Mass	Identify Protein	Protein sequence coverage	Score	Index
phoA-L 45-50*	MASCOT		Poly (A) polymerase I	50	28	-
	Protein Prospector	-	N/A	-	-	-
	PROFOUND	-	N/A	-	-	-
phoA-M 15-20*	MASCOT	11	30S ribosomal protein S10	46	68	gi|487397367
		28	Fumarate and nitrate reduction regulatory protein	44	43	-
			ribosomal protein S10	34	69	gi|487397367
		29	Nickel import ATP-binding protein NikE	30	48	-
	Protein Prospector		30S ribosomal protein S10	44.7	2003	387038
		9007	50S ribosomal protein L28	59	1090	356052
	PROFOUND	-	N/A	-	-	-
phoA -N 15-20*	MASCOT	16	YHBP_ECO7I	74	31	-
	Protein Prospector	32058	Putative type II secretion system L-type protein YghE	19.2	159	527985
	PROFOUND	-	N/A	-	-	-

Table 3: *Proteins identified from pull down assay of PhoA mRNA using MALDI-MS identification.*

Discussion

Protein secretion is an essential, and therefore evolutionarily conserved, cellular process. The importance of this process is further displayed by the finding that there are of up to eight different protein secretion systems in prokaryotes [43]. By far, the most conserved and best recognized as the general secretory system is the Sec-dependent system. In prokaryotes, the signal peptide hypothesis elegantly maps how pre-secretory proteins are recognized by the cell, targeted to the Sec translocon within the inner membrane, and eventually exported outside the inner membrane. In certain studies, however, this hypothesis appeared not to be held as some experimental find-

ings, could not be explained through this hypothesis [12,44]. Recent studies on the secretion of the effectors of Type III secretion in *Yersinia* species have provided a plausible alternate hypothesis whereby the pre-secretory proteins are recognized and tagged for secretion through signals in the 5' region of the proteins' mRNA rather than through signals in the protein's amino acid signal sequence [12]. This became known as the mRNA hypothesis. In an effort to explain the experimental observations where the signal peptide hypothesis did not hold for certain Sec-dependent secreted proteins, I hypothesized that in addition to recognizing the signal peptide, the SRP also requires simultaneous recognition of the 5' mRNA of the secretory protein. I called this the alternate hypothesis.

In this study I examined the possibility that the Sec-dependent secreted proteins are targeted to the Sec system via signatures in the mRNA structure rather than/or in addition to, signatures (signal peptides) in the protein structure. I looked at Sec targeting indirectly by establishing interactions between the mRNA test constructs and the SRP equivalent in *E.* coli lysates, including the fifty four homologue (Ffh) cytoplasmic factor [1,5,42,46,56]. Association between the test mRNA constructs and Ffh or other cytoplasmic factors was followed using a pull down assay. In this assay, the mRNA was generated using *in vitro* transcription reactions of DNA segments representing the 5' UTR of secreted (test) and cytoplasmic (control) gene products as well as segments representing the signal peptide or its equivalent in a cytoplasmic protein (N-terminal 25 amino acids). These transcripts were then used to pull down cytoplasmic factors from total cell lysates from *E. coli* that are then identified using mass spectrometry. Using this approach, several cytoplasmic factors were pulled down and identified (Table 2 and 3).

The surA protein was used as the secretory model protein. SurA is formally identified as a periplasmic propyl isomerase chaperone of *E. coli*, which is required for proper biogenesis of outer membrane proteins [4,48]. This protein contains a typical signal sequence in its N-terminus and could potentially provoke further insight into characterization of the RNA binding proteins (RBPs) in *E. coli*. Moreover, it could open new avenues to understand the function of RBPs in the translocation machinery that is located in the cytoplasmic membrane. The 3-isopropylmalate dehydratase enzyme was used in this study as a control for non-secreted proteins, given that it is a cytoplasmic enzyme. This enzyme is actually involved in the biosynthesis of the amino acid leucine [14,54].

Factors identified through the pull-down assay

Pull-down assays using the synthetic mRNAs for surA and isodH followed by analysis of enriched proteins by SDS-PAGE identified several different bound proteins. Hence, there does not seem to be any protein that specifically binds to one mRNA over the other. Following optimization of the assay, distinct SDS-PAGE patterns were shown to distinguish binding between the two mRNA baits. In order to establish the identity of these factors, the bands were excised for in-gel trypsin digest followed by MALDI MS identification.

Of the proteins identified from the pull-down assay with surA mRNA, proteins corresponding to the *Staphylococcus aureus* SRP protein and *S. aureus* FtsY-like protein are perhaps the most curious. These proteins, which have been discovered in Gram-positive as well as Gram-negative bacteria, are involved in membrane proteins targeting and eventually to the co-translational secretion [46]. Repeated experiments with this surA mRNA construct did not show any specific association with *E. coli* SRP.

Remarkably, other proteins identified in this assay include the 3-isopropylmalate dehydratase enzyme; the fused protein chain initiation factor 2 (IF-2), DEAD/DEAH box helicase protein and Acetyltransferase GNAT family protein. Some of these proteins, as in the case of the isodH mRNA pull down assay, have established functions related to mRNA unfolding, processing, translation control, or decay. Particularly in *E. coli*, most of the proteins would be expected to bind the mRNA of the 3-isopropylmalate dehydratase enzyme as those are the ones involved in the critical steps in normal translation of mRNA [16,29] (Table 2).

Interestingly, MALDI MS identified the presence of the 3-isopropylmalate dehydratase enzyme (a 39 kDa protein) in extracts pulled down by the mRNA (Table 2). This result could potentially provide insight into the fact that this type of enzyme might belong to any of the translation factors. Thus, we can establish that this type of binding interaction involves the regulation of the mRNA product.

The fused protein chain initiation factor 2 (IF-2) has been identified from MALDI MS analysis in table 2. It is a GTPase that has a significant role in promoting the initiation of protein synthesis in prokaryotes. IF2 enhances the binding of 30S ribosomal subunit to the initiator tRNA and thus controls the delivery of tRNA into the ribosome. This result would actually support the interactions between translation factors and RNA sequences of cytoplasmic proteins, which is believed to be subsequently involved in protein biosynthesis [31].

In addition to IF-2, DEAD/DEAH box helicase proteins have been identified. This protein family contributes to ribosome biogenesis, RNA metabolism, mRNA decay and possibly translation initiation in *E. coli*. Most of DEAD box proteins participate in the assembling RNA or ribonucleoprotein (RNP) structure *in vitro*. This is related to its function as ATP-dependent RNA helicases and RNA-dependent ATPases [15]. The presence of DEAD/DEAH box helicases from the pull-down assay is logical according to their function. It has been proven that the Acetyltransferase GNAT family proteins in bacteria appear to be involved in resistance to some antibiotics. However, there is no previously identified function for these protein types in mRNA translation or processing [53]. I can conclude that participation of the GNAT in mRNA recognition is not known.

Some of the other proteins identified, however, are interesting in that they appear only in the pull-down assay using the surA transcript and are not present in the isodH mRNA pull-downs. For example, the Nucleoid-associated protein, YbaB, specifically interacts with the surA mRNA and has been previously identified as a factor that binds DNA and thus changes its conformation. Additionally, this protein appears to be involved in regulating gene expression [8]. Proteins that bind DNA have been found also to bind RNA in many instances as has been demonstrated for the DNA binding protein H-NS [6].

Different proteins identified in pull down assay with the IsodH mRNA include DNA-dependent helicase, Lhr, which is a SF2 helicase and one of the longest known proteins in *E. coli* containing motifs from the helicase superfamily II [36,41]. Further study proved that this polypeptide also contains an amino region similar to the DEAD family helicases [41]. Ordonez [36] have shown that some of the helicases have established functions related to RNA turnover, processing, translation control, and ribosome biogenesis.

In a similar manner the Multifunctional conjugation protein TraI (Table 2) has been identified as a DNA helicase I. Furthermore, TraI has been shown to have a major role in binding and cleaving single-stranded DNA oligonucleotides containing an oriT sequence in plasmid transfer experiments [10,50]. TraI also functions in the initiation and/or termination of plasmid transfer [10].

Different transcripts construct (5' UTRs of phoA and gMP genes)

It is quite possible that binding of a translation factor is dependent not only on the 5' translated region of the mRNA but in fact requires sequences in the untranslated region (UTR) [3]. Direct evidence supporting the importance of the UTR comes from the recent observation that the ribosomal protein S1 [3,13] which is essential for *in vivo* translation in *E. coli* [13] can bind to an 11 nucleotides region in the mRNA immediately upstream of the Shine-Dalgarno (SD) sequence [3,45]. The SD sequence is a short sequence of mRNA that interacts with anti-SD sequences in the 3' end of 16S rRNA which is involves in the initiation of protein translation [3,19,28]. The SD sequence-S1 protein interaction further plays a significant role in effecting gene expression [3]. Additionally, it has been demonstrated that there is a parallel approach in *Pseudomonas aeruginosa* that further supports a role for the 5' UTR in *E. coli* translation regulation. This evidence indicates that S1 protein preferentially binds within the first 78 nucleotides of the 5' UTR of the *rpoS* mRNA and thus may contribute to the translational regulation of *rpoS* mRNA [47].

Interestingly, since the UTR sequence is considered to be important for targeting the location of mRNA to specific cellular locations via interactions with specific proteins, it seems likely that protein binding to the UTR of different transcripts may have other functional roles. It was therefore of interest to establish an artificial transcript that includes the 5' UTR of phoA and gMP genes for use in the pull-down experiments. Using this construct as bait I was able to pull down a protein, which has been previously shown to be a polyadenylation-regulating protein. SDS-PAGE shows a unique 50 kDa band that was present in the pull-down experiment using the phoA mRNA bait but

not in the controls (Figure 4A). The band was identified as the poly (A) polymerase I enzyme (PAPI) that is actually responsible for the addition of adenylyl residues to the 3' hydroxyl termini of new mRNA molecules. The mRNA bait constructs that were used in these experiments do not have poly A tails since the in- *vitro* transcription reaction I used does not generate poly A tails. However, it is possible that the PAPI proteins are able to bind to other parts of the mRNA. For example, it is possible that the poly (A) polymerase I protein it is able to binds first to the RNA and then initiate the polyadenylation process [17]. Hence, these results cannot exclude the possibility that sequence in the UTR of mRNA might be able to bind several proteins that are necessary for the different functions (Table 3).

The S10 protein of *Escherichia coli* is a member of the ribosomal protein (r-proteins) group that is located in the head of the 30 S subunit of the ribosome [24]. A major function of S10 is its ability to mediate binding of tRNA to the ribosomes [57]. Studies have also reported that the S10 protein has a role in anti-termination of the transcription process [7,24]. It has been shown that the 50S ribosomal protein L28 cross-links to two distinct RNA regions in *E. coli* [37,51]. Moreover, this protein has a critical role in ribosome assembly and the absence of L28 also prevented the synthesis of the ribosome [26]. Further experiments indicated that the function of ribosomal proteins is not only restricted to the ribosome but that it has another role such as mediating the association of ribosomal protein L22 during export of a secreted virulence factor antigen 43 (AG 43) in *E.coli* [55]. Additionally, it has been verified there is a connection between the physiological roles of ribosomal proteins and RNase since it has been shown that RNase co-migrates with 30S ribosomal subunit [27]. Our results might promote the idea that there are additional functions of ribosomal protein in *E. coli.*

The putative type II secretion system L-type protein YghE is another interesting protein identified in the pull-down assays (Figure 5B, spot N). YghE belongs to the general secretion pathway (GSP L family) that locates in the inner membrane. This protein family is involved in a type II secretion system for the export of proteins [11]. The MALDI MS identification of pulled down proteins further identified the Fumarate and nitrate reduction (FNR) regulatory protein that is recognized as a global transcription factor. FNR regulates a large family of genes in response to environmental conditions and also appear to bind DNA [20].

Altogether I was unable to pull down secretion-dependent factors that recognize and bind selectively to the mRNA of secreted proteins. The proteins that were identified are typically associated within mRNA processing. Some of these proteins have been also pulled down using similar methodologies in different laboratories. Maier., *et al*. (2008) demonstrated that the SRP - protein KdpD interaction in *Escherichia coli* is required for KdpD protein targeting. The complex was further confirmed using pull down experiments

Conclusion

In our study a direct interaction between mRNAs encoding secreted proteins and the *E.* coli SRP equivalent (Ffh) was pursued using pull down assays. Following extensive optimizations and modifications of these experiments, the Ffh protein (the *Escherichia coli* SRP homolog) could not be isolated from cytoplasmic extracts of *E. coli* with the pull-down assays. One interesting finding however was that the mRNA of the IsodH protein was pulled down using its cognate mRNA transcript as bait. This implies a role for this enzyme in regulating its own levels in the cell by binding to and potentially modulating its translation. Other factors involved in DNA and RNA binding were also isolated and include RNase and ribosomal proteins, amongst others. It can therefore be concluded that under these experimental conditions, the mRNA hypothesis for targeting protein secretion could not be supported.

Conflict of Interest

The authors declare that there is no conflict of interest.

Bibliography

1. Angelini S., *et al*. "Membrane binding of the bacterial signal recognition particle receptor involves two distinct binding sites". *The Journal of Cell Biology* 174 (2006): 715-724.

2. Beckwith J. "The Sec-dependent pathway". *Research in Microbiology* 164 (2013): 497-504.

3. Berg L., *et al*. "The expression of recombinant genes in *Escherichia coli* can be strongly stimulated at the transcript production level by mutating the DNA-region corresponding to the 5'-untranslated part of Mrna". *Microbial Biotechnology* 2 (2009): 379-389.

4. Bitto E and McKay DB. "The periplasmic molecular chaperone protein SurA binds a peptide motif that is characteristic of integral outer membrane proteins". *The Journal of Biological Chemistry* 278 (2003): 49316-49322.

5. Braig D., *et al*. "Signal sequence-independent SRP-SR complex formation at the membrane suggests an alternative targeting pathway within the SRP cycle". *Molecular Biology of the Cell* 22 (2011): 2309-2323.

6. Brescia CC., *et al*. "The DNA binding protein H-NS binds to and alters the stability of RNA *In vitro* and *In vivo*". *Journal of Molecular Biology* 339 (2004): 505-514.

7. Brodersen DE and Nissen P. "The social life of ribosomal proteins". *The FEBS Journal* 272 (2005): 2098-2108.

8. Cooley AE., *et al*. "DNA-binding by *Haemophilus influenzae* and *Escherichia coli* YbaB, members of a widely-distributed bacterial protein family". *BMC Microbiology* 9 (2009): 1-8.

9. Cuia J., *et al*. "Generation of transgenic zebrafish with liver-specific expression of EGFP-Lc3: A new *in vivo* model for investigation of liver autophagy". *Biochemical and Biophysical Research Communications* 2 (2012): 268-273.

10. Dna S., *et al*. "DNA Recognition by F Factor TraI36: Highly Sequence-Specific Binding of Single-Stranded DNA". *Biochemistry* 40 (2001): 11586-11595.

11. Filloux A. "The underlying mechanisms of type II protein secretion". *Biochimica et Biophysica Acta* 1694 (2004): 163-179.

12. Habyarimana F and Ahmer BMM. "More evidence for secretion signals within the mRNA of type 3 secreted effectors". *Journal of Bacteriology* 195 (2013): 2117-2118.

13. Hajnsdorf E and Boni IV. "Multiple activities of RNA-binding proteins S1 and Hfq". *Biochimie* 94 (2012): 1544-1553.

14. He Y., *et al*. "Functional specification of Arabidopsis isopropylmalate isomerases in glucosinolate and leucine biosynthesis". *Plant and Cell Physiology* 51 (2010): 1480-1487.

15. Iost I and Dreyfus M. "DEAD-box RNA helicases in *Escherichia coli*". *Nucleic Acids Research* 34 (2006): 4189-4197.

16. Kaczanowska M and Rydén-Aulin M. "Ribosome biogenesis and the translation process in *Escherichia coli*". *Microbiology and Molecular Biology Reviews* 71 (2007): 477-494.

17. Kushner SR and Mohanty BK. "Bacterial/archaeal/organellar polyadenylation". *Wiley Interdisciplinary Reviews-RNA* 2 (2011): 256-276.

18. Kudva R., *et al*. "Protein translocation across the inner membrane of Gram-negative bacteria: the Sec and Tat dependent protein transport pathways". *Research in Microbiology* 164 (2013): 505-534.

19. Laursen BS., *et al*. "Initiation of Protein Synthesis in Bacteria". *Microbiology and Molecular Biology Reviews* 69 (2005): 101-123.

20. Lee DJ., *et al*. "Analysis of interactions between Activating Region 1 of *Escherichia coli* FNR protein and the C-terminal domain of the RNA polymerase a subunit: use of alanine scanning and suppression genetics 37 (2000): 1032-1040.

21. Lee VT and Schneewind O. "Protein secretion and the pathogenesis of bacterial infections". *Genes and Development* 15 (2001): 1725-1752.

22. Lehnhardt S., *et al*. "Modulation of the effects of mutations in the basic region of the OmpA signal peptide by the mature portion of the protein". *The Journal of Biological Chemistry* 263.1988 (1988): 10300-10303.

23. Low KO., *et al*. "Optimisation of signal peptide for recombinant protein secretion in bacterial hosts". *Applied Microbiology and Biotechnology* 97 (2013): 3811-3826.

24. Luo X., *et al*. "Structural and functional analysis of the *E. coli* NusB-S10 transcription antitermination complex". *Molecular Cell* 32.6 (2009): 791-802.

25. Maier KS., *et al*. "An amphiphilic region in the cytoplasmic domain of KdpD is recognized by the signal recognition particle and targeted to the *Escherichia coli* membrane". *Molecular Microbiology* 68 (2008): 1471-1484.

26. Maguire BA and Wild DG. "The roles of proteins L28 and L33 in the assembly and function of *Escherichia coli* ribosomes *In vivo*". *Molecular Microbiology* 23 (1997): 237-245.

27. Malecki M., *et al*. "Characterization of the RNase R association with ribosomes". *BMC Microbiology* 14 (2014): 1-9.

28. Malys N. "Shine-Dalgarno sequence of bacteriophage T4: GAGG prevails in early genes". *Molecular Biology Reports* 39 (2012): 33-39.

29. Marintchev A and Wagner G. "Translation initiation: structures, mechanisms and evolution". *Quarterly Reviews of Biophysics* 37 (2004): 197-284.

30. Michaelis S., *et al*. "Effects of signal sequence mutations on the kinetics of alkaline phosphatase export to the periplasm in *Escherichia coli*". *Journal Bacteriology* 167 (1986): 160-167.

31. Milon P., *et al*. "The ribosome-bound initiation factor 2 recruits initiator tRNA to the 30S initiation complex". *EMBO Reports* 11 (2010): 312-316.

32. Mori H and Ito K. "The Sec protein-translocation pathway". *Trends in Microbiology* 9 (2001): 494-500.

33. Nevo-Dinur K., *et al*. "Translation-independent localization of mRNA in *E. coli*". *Science* 331 (2011): 1081-1084.

34. Nijtmans LGJ., *et al*. "Blue Native electrophoresis to study mitochondrial and other protein complexes". *Methods* 26 (2002): 327-334.

35. Okita TW and Choi SB. "mRNA localization in plants: targeting to the cell's cortical region and beyond". *Current Opinion in Plant Biology* 5.6 (2002): 553-559.

36. Ordonez H and Shuman S. "Mycobacterium smegmatis Lhr Is a DNA-dependent ATPase and a 3'-to-5' DNA translocase and helicase that prefers to unwind 3'-tailed RNA:DNA hybrids". *The Journal of Biological Chemistry* 288 (2013): 14125-14134.

37. Osswald M., *et al*. "Localization of a series of RNA-protein cross-link sites in the 23S and 5S ribosomal RNA from *Escherichia coli*, induced by treatment of 50S subunits with three different bifunctional reagents". *Nucleic Acids Research* 18 (1990): 6755-6760.

38. Papanikou E., *et al*. "Bacterial protein secretion through the translocase nanomachine". *Nature Reviews Microbiology* 5 (2007): 839-851.

39. Peterson JH., *et al*. "An unusual signal peptide extension inhibits the binding of bacterial presecretory proteins to the signal recognition particle, trigger factor, and the SecYEG complex". *The Journal of Biological Chemistry* 281 (2006): 9038-9048.

40. Puziss JW., *et al*. "Analysis of mutational alterations in the hydrophilic segment of the maltose-binding protein signal peptide". *Journal of Bacteriology* 171 (1989): 2303-2311.

41. Reuven NB., *et al*. "The Gene for the Longest Known *Escherichia coli* Protein Is a Member of Helicase Superfamily II". *Journal of Bacteriology* 177 (1995): 5393-5400.

42. Rosch JW., *et al*. "The signal recognition particle pathway is required for virulence in Streptococcus pyogenes". *Infection and Immunity* 76 (2008): 2612-2619.

43. Saier MH. "Protein Secretion Systems in Gram-Negative Bacteria". *Microbe* 1 (2006): 414-419.

44. Samander H., *et al*. "Prediction of Sec-dependent Secreted Proteins Based on mRNA Structure of Signal Peptides". *World Congress in Computer Science, Computer Engineering, and Applied Computing* (2013): 1-7.

45. Sengupta J., *et al*. "Visualization of protein S1 within the 30S ribosomal subunit and its interaction with messenger RNA". *Proceedings of the National Academy of Sciences of the United States of America* 98 (2001): 11991-11996.

46. Schneewind O and Missiakas DM. "Protein secretion and surface display in Gram-positive bacteria". *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 367 (2012): 1123-1139.

47. Sevo M., *et al*. "Ribosomal Protein S1 Specifically Binds to the 5 ′ Untranslated Region of the Pseudomonas aeruginosa Stationary-Phase Sigma Factor rpoS mRNA in the Logarithmic Phase of Growth". *Journal of Bacteriology* 186 (2004): 4903-4909.

48. Sklar JG., *et al*. "Defining the roles of the periplasmic chaperones SurA, Skp and DegP in *Escherichia coli*". *Genes and Development* 21 (2007): 2473-2484.

49. Sorg JA., *et al*. "Substrate recognition of type III secretion machines--testing the RNA signal hypothesis". *Cellular Microbiology 7* (2005): 1217-1225.

50. Street LM., *et al*. "Subdomain organization and catalytic residues of the F factor TraI relaxase domain". *Biochimica et Biophysica Acta - Proteins and Proteomics* 1646 (2003): 86-99.

51. Urlaub H., *et al*. "Protein-rRNA binding features and their structural and functional implications in ribosomes as determined by cross-linking studies". *The EMBO Journal* 14 (1995): 4578-4588.

52. Van Der Wolk JP., *et al*. "PrlA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA-SecY interaction during the initiation of translocation". *The EMBO Journal* 17 (1998): 3631-3639.

53. Vetting MW., *et al*. "Structure and functions of the GNAT superfamily of acetyltransferases". *Archives of Biochemistry and Biophysics* 433 (2005): 212-226.

54. Velíšek JAN and Cejpek K. "Biosynthesis of Food Constituents: Amino Acids: 2. The Alanine-Valine-Leucine, Serine-Cysteine-Glycine and Aromatic and Heterocyclic Amino Acids Groups-a Review". *Czech Journal of Food Science* 24 (2006): 45-58.

55. Yap MNF and Bernstein HD. "Mutations in the *Escherichia coli* ribosomal protein L22 selectively suppress the expression of a secreted bacterial virulence factor". *Journal of Bacteriology* 195 (2013): 2991-2999.

56. Yosef I., *et al*. "*Escherichia coli* SRP, Its Protein Subunit Ffh, and the Ffh M Domain Are Able To Selectively Limit Membrane Protein Expression When Overexpressed". *Mbio* 1 (2010): 1-9.

57. Zurawski G., *et al*. "Structure of the *Escherichia coli* S10 ribosomal protein operon". *Nucleic Acids Research* 13 (1985): 4521-4526.

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