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Functional interactions of metalloprotein YbeY, involved in ribosomal metabolism, with the putative metal efflux protein YbeX

Master's Thesis

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Functional Interactions of metalloprotein YbeY, involved in ribosomal metabolism, with the putative metal efflux protein YbeX

YbeY is a putative ribosomal endoribonuclease which has been implicated, among other things, to be involved in quality control of 70S ribosomes, in 17S pre-rRNA maturation and in ribosomal degradation. However, controversy reigns over its mode of action, substrates, co-factors, and interaction partners. Proposed interactors of YbeY include ribosomal protein S11, Era, YbeZ, and SpoT.
In many bacteria *ybeY* is located in the *ybeZYX-Int* operon, where *ybeZ* encodes a PhoH subfamily protein with NTP hydrolase domain and *ybeX* encodes a putative Cobalt/Magnesium efflux protein. Depletion of YbeY and YbeX have largely overlapping phenotypes, including accumulation of 17S pre-rRNA and an approximately 1 kb 16S rRNA cleavage product, sensitivity to heat shock, and to the protein synthesis inhibitors chloramphenicol and erythromycin. Overexpression of the YbeY partially rescues the some of the phenotypes of *ΔybeX*. Taken together our results indicate a functional interaction between *ybeY* and *ybeX*.
Keywords: YbeY, rRNA processing, ribosomes

CERCS code: P310 – Proteins, enzymology; B230 – Microbiology, bacteriology, virology, mycology

Metalloproteiini YbeY funktsionaalsed koostoimed ribosoomses ainevahetuses, oletatava metalli efflux-valguga YbeX

YbeY on oletatav ribosomaalne endoribonukleaas, mis on muu hulgas ka seotud 70S ribosoomide kvaliteedikontrolliga, 17S-i rRNA küpsemise ja ribosoomide lagundamisega, kuigi siin on palju vaidlusi töömehhanismi, substraatide, kaasfaktorite ja nende koosmõju kohta. YbeY interaktsioonid on seotud selliste ribosomaalsete valgudega nagu S11, Era, YbeZ ja SpoT.

Paljudes bakterites *ybeY* paikneb *ybeZYX-Int* operonis, kus *ybeZ* kodeerib PhoH alamperekonna valku NTP hüdrolaasi domeeniga ja *ybeX* kodeerib oletatava koobalti / magneesiumi väljavoolu valgu. YbeY ja YbeX puuduse olukorras tekivad suuresti kattuvad fenotüübid, kaasaarvatud 17S-i rRNA ja liigikaudu 1 kb 16S-i rRNA lõhustamisprodukti kogunemine, tundlikkus kuumašoki ja valgusünteesi inhibiitorite kloramfenikooli ja erütromütsiini suhtes. YbeY`i üleekspressioon saab osaliselt kompenseerida mõneid *ΔybeX* tüüvide fenotüübi. Kokkuvõttes meie tulemused näitavad funktsionaalset interaktsiooni *ybeY* ja *ybeX* vahel.

Märksõnad: YbeY, rRNA töötlemine, ribosoomid

CERCS kood: P310 – Proteiinid, ensüümid; B230 – Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

CONTENTS

ABBREVIATIONS

rRNA – ribosomal ribonucleic acid

nt – nucleotide

mRNA – messenger ribonucleic acid

tRNA – transfer ribonucleic acid

CAM – Chloramphenicol

ERYTH - erythromycin

EDTA – Ethylenediaminetetraacetic acid

SDS – Sodium Dodecyl Sulphate

EtOH – ethanol

EtBr – ethidium bromide

CBS – cystathionine beta synthase

AMP – Adenosine monophosphate

ATP – Adenosine triphosphate

TET– Tetracycline

stat- Stationary growth phase

exp – Exponential growth phase

INTRODUCTION

Cell is defined as the unit of life, and cell itself defines life. Ribosome is an organelle that is present in all living organisms, a highly packed ribonucleoprotein complex composed of 3-4 RNA (rRNA) species and of scores of ribosomal proteins.

The biogenesis of ribosome is a rather complex macromolecular assembly process and it is regulated from transcription of ribosomal RNA to synthesis of ribosomal proteins, which start folding on the rRNA transcript while the transcription is ongoing. Once the precursor subunits are made the precursor rRNA species are trimmed with ribonucleases to complete the maturation of the subunits. This is where YbeY protein and its operon *ybeZYX-lnt* comes into our attention. YbeY is a putative endoribonuclease that has been shown to play a role in the processing of the 3`end of 16S ribosomal RNA of both *Escherichia coli* (Jacob et al., 2013) and *Bacillus subtilis* (Baumgardt et al., 2018).

In prokaryotes UPF0054 protein family is highly conserved and *ybeY* belongs to this family (Rasouly et al., 2009). *ybeY* is found in almost all sequenced bacterial genomes, and it is among the predicted minimal bacterial genome set (Gil et al., 2004). The *ybeY* is a conserved gene found in a conserved operon called *ybeZYX-lnt*. The *ybeZYX-lnt* operon is required for normal type 3 secretion system in enterohemorrhagic *Escherichia coli* (McAteer et al., 2018). In the *ybeZYX-Int* operon, *ybeZ* encodes a PhoH subfamily protein with NTP hydrolase domain and *ybeX* encodes a putative Cobalt/Magnesium efflux protein. The maturation of ribosome and ribosomal components are membrane dependent. Although the location of YbeX in cell is still elusive (it might be a cytosolic protein or a transmembrane protein), its role in metal regulation could be the key to unlocking the role of metalloprotein YbeY together with other operon proteins.

In this thesis litreature review, I will give a very brief overwiev of the prokaryotic ribosome and the processing of the ribosomal RNA which is trimmed by ribonucleases. I will try to cover the work, which has already been done on YbeY and avaliable in the litreature. And also give a full account of published information on the *ybeZYX-lnt* operon genes *ybeZ* and *ybeX*.

1. LITERATURE REVIEW

1.1 Structure of the prokaryotic ribosome

The ribosome is responsible for protein synthesis in all living organisms and consists of two unequal-sized subunits. In bacteria the large subunit (50S) has a molecular weight of 1.6 MDa while the small subunit (30S) has a weight of 0.9 MDa (Londei et al., 1983). The size of ribosomal particles is measured in Svedberg`s units, which is a unit of sedimentation velocity (Griffiths et al., 2002).

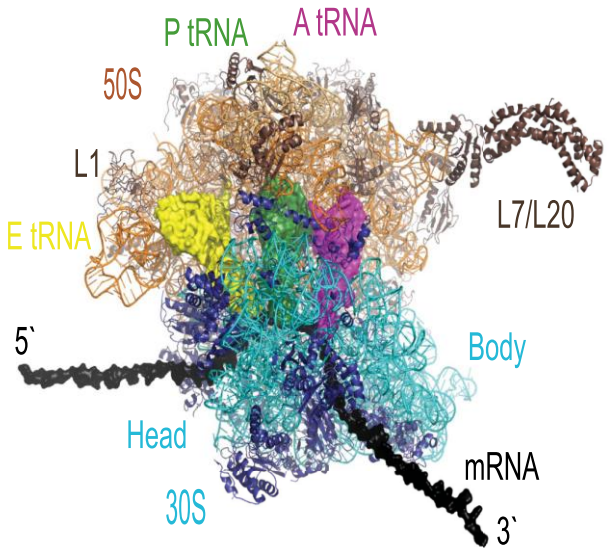


Figure 1 | Top view of 70S ribosome on the mRNA (shown in black). E-site (yellow), P-site (green) and A-site (purple) tRNAs are shown. 30S subunit is shown with blue (head and body). The large ribosomal subunit 50S is shown in brown. The structure was adopted from (Schmeing and Ramakrishnan, 2009). The large ribosomal subunit proteins L7/L12, and L1 stalks, which are functionally important in translocation and binding, are shown (Ramakrishnan, 2002).

The 50S subunit consists of 23S RNA (~2.9 kb), 5S RNA (~0.12 kb) and about 30 ribosomal proteins while the 30S subunit consists of one rRNA species 16S RNA (~1.5 kb) and about 20 ribosomal proteins. Bacterial functional ribosomes have a size of 70S (more than 2.3 MDa), and are associated from the 30S and 50S subunits into functional ribosomes to accomplish protein synthesis in the cell (Wilson and Nierhaus, 2007).

1.2 Ribosomal RNA (rRNA) and its processing

In *Escherichia coli* three rRNAs (16S, 23S, 5S) are co-transcribed from seven near-identical rRNA operons as a single molecule (one of the seven, rrnB, is shown in Figure 2). Having seven copies of the rRNA operon has the double advantage of being able to make a lot of ribosomes quickly, and of allowing regulating the ribosome biogenesis.

The pre-transcripts require multiple RNase cleavages to produce the mature rRNAs (shown in **Figure 3**). The first processing step is carried out by RNase III, which cleaves in the double-stranded regions flanking 23S and 16S rRNA (Li and Deutscher, 2004).

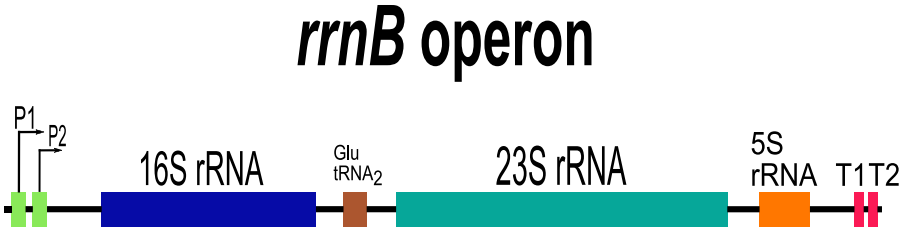


Figure 2 | One of the seven ribosomal operon, *rrnB* general structure in *E. coli*. The promotors (P1, P2) are shown in green. Coding regions of mature 16S rRNA (blue), 23S rRNA(green), and 5S rRNA are shown together with the spacer tRNA (brown), and terminators (T1,T2, red).

Ribosomal RNA is the most complex RNAs made in *E. coli*. The process requires multiple exoribonucleases are known; RNase II, RNaseR, RNase PH, PNPase (polynucleotide phosphorylase), RNase III, RNase E, RNase T, ORNase (oligoribnuclease, not shown), and many other unidentified RNases (Figure 3). The trimming 3′ end of 16S rRNA is thought to be generated by an endonucleolytic activity (Li and Deutscher, 2004), and recently YbeY has been claimed to be the endoribonuclease for the processing (Baumgardt et al., 2018; Jacob et al., 2013). The latest theory for 3`-end of 16S rRNA processing (Sulthana and Deutscher, 2013) suggests that four 3` to 5` exoribonucleases RNase II, R,PH and PNPase do it together with GTPase Era and endoribonuclease YbeY (Ghosal et al., 2018).

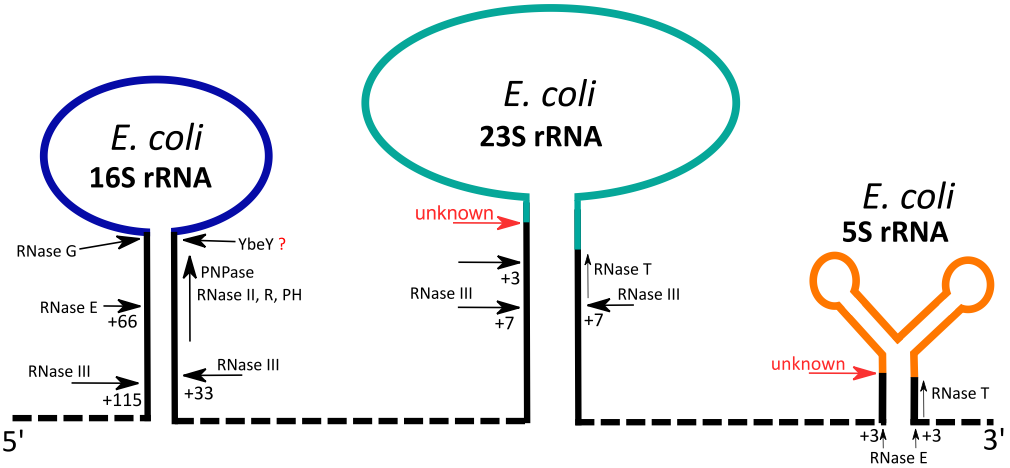


Figure 3 | Processing of ribosomal RNA precursor transcripts. The required RNase`s cleavages for production of mature 16S rRNA(blue), 23S rRNA (green), and 5S rRNA (orange) is shown. The cleavage sites are indicated with arrows, and the name of the ribonuclease given. Cleave site distance from the mature rRNA is indicated with numbers (etc. +3). After an important step carried out by the RNase III (four cleavage sites are shown), the 17S rRNA, pre-23S rRNA and 9S rRNA intermediates (shown with bold black line together with the colourful mature rRNA regions) are made. The predicted YbeY cleavege site is shown (elusive site is annotated with red question mark). The trimmer for 5`end of 23S rRNA and 5SrRNA are still unknown (marked with red arrows). This figure was modified from (Baumgardt et al., 2018).

1.3 Multi-functional metalloprotein YbeY

The *ybeY* is a highly conserved member of *ybeZY* operon in both gram negative and positive bacteria (Anantharaman and Aravind, 2003) (Supp Fig. S5). Sequence alignment of the YbeY orthologs shows its similarity to the Zinc-like metallopeptidases with its conserved 3 histidine residue (Anantharaman and Aravind, 2003). The first attempt to purify *Escherichia coli* YbeY homologue, and its crystallography studies shed on its metal dependent hydrolase structure with three histidine molecule holding a metal in the cleft of the protein, and the X-ray Fluorescence spectroscopy revealed the metal to be nickel (Zhan et al., 2005).

The processing of 3´ end of 16S rRNA involves multiple exoribonucleases shown in Figure 3 (Sulthana and Deutscher, 2013). YbeY is involved in the processing of the 16S rRNA 3`- end in *E. coli* and in *B. subtilis* (Baumgardt et al., 2018; Jacob et al., 2013). The *E. coli* YbeY depletion influences the processing of the 16S rRNA 3`-end, and result in defects in ribosome assembly (Davies et al., 2010). Ribosomal profile analysis of the *ybeY* deletion mutant strain points to defects in translation (Rasouly et al., 2009). In many bacteria; *Sinorhizobium meliloti* (Davies and Walker, 2008), *Vibrio cholera* (Vercruysse et al., 2014), *Agrobacterium tumefaciens* (Möller et al., 2019), *Corynebacterium glutamicum* (Maeda et al., 2017), and

even in *Arabidopsis thaliana* chloroplast (Liu et al., 2015), studies suggest a conserved role for YbeY to be required for proper ribosomal RNA processing, and its depletion causes many defects in living organisms, to such extent that in some cases (*B. subtilis*) it is an essential gene (Baumgardt et al., 2018).

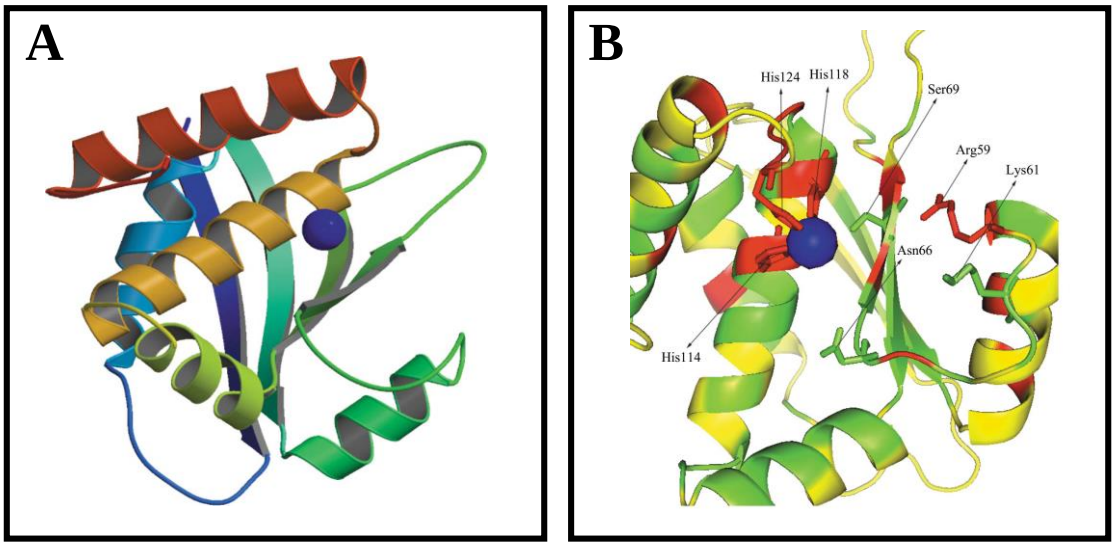


Figure 4 | Crystal structure of YbeY. (A) Tertiary structure of protein YbeY. The overall protein structure consists of six α-helices and four β-strands in a β- α - α -β- α -β-β- α - α - α fold. (B) The cleft structure of YbeY is presented in secondary structure where the metal ion (blue sphere) is surrounded with 3 histidine residue (His114, His124, His118) and highly conserved arginine (Arg59). Red coloured residues in Panel B represent the high conserved regions. The structures were adopted from Zhan and his colleagues (Zhan et al., 2005).

YbeY structure reveal it's a metal binding protein with tetrahedral geometry, shown in **Figure 4** (Zhan et al., 2005). YbeY endoribonuclease activity need its metal ion to function (Jacob et al., 2013), and addition of metal ion (in µM ratio) to the YbeY enzymatic reaction (synthetic RNA oligoes used as substrate) has an effect of inhibition (calcium ions) or influence (Mg^{2+},Mn^{2+} and Ca^{2+}) in a different manner on cleavage efficiency (Saramago et al., 2017). On the other hand, endoribonuclease YbeY failed to exhibit nucleolytic activity on the 3`-end of 16S rRNA *invitro* when purified pre-SSU (precursor small subunits, pre-30S) were mixed with YbeY with/without RNase G (Gupta and Culver, 2014). Purified YbeY homolog from *S. meliloti* and *E. coli* does not exhibit any nucleolytic effect *invitro* on double stranded RNA (Jacob et al., 2013; Möller et al., 2019).

Interestingly, purified YbeY act on the single stranded RNA (ssRNA) at different labs with different action; YbeY cleave the substrate ssRNA in earlier work (Jacob et al., 2013) but the latest repeated experiment bring out a degradation of the same ssRNA substrate under the same experimental set up (Smith et al., 2018).

YbeY is a single stranded specific endoribonuclease together with RNase R able to degrade only 70S ribosomes which are purified from *ybey* deletion mutant *E. coli* and *Vibrio cholera* (Vercruysse et al., 2014) strains, meaning that the ribosomes are defective and the defect is at the 30S level. Ribosomes purified from wild type *E. coli* kasugamycin treated cells (Jacob et al., 2013) act as a substrate for YbeY/RNase R degradation. The *ybeY* is a highly conserved gene among bacteria, and its homologs in *Vibrio cholera*, *Pseudomonas auruginosa*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* seems to compensate for the strong phenotype in *E. coli* (Vercruysse et al., 2014) which claim a conserved role in ribosomal RNA processing. Studies on ybeY has key to understanding the ribosomal quality control and maturation, and ybeY suggest a potential target for antibacterial drugs.

1.4 ATP-binding protein YbeZ (phoH-like protein)

A strong interaction between metalloprotein YbeY and a putative ATPase YbeZ has already been shown via bacterial two-hybrid system (Vercruysse et al., 2016). Phosphate starvation regulated *E. coli* PhoH-like protein YbeZ is a protein of unknown function. YbeZ express phosphatase activity and considered to be a putative RNA helicase, as the phoH gene encodes a member of the helicase-like superclass of the P-loop NTPase fold (Kazakov et al., 2003; Wanner and Latterell, 1980). Other than this, we know very little about the function of YbeZ.

E. coli PhoH homologs are present in different organisms, but it is unclear if this is a homology or an analogy. In *Bacillus subtilis* and many other gram positive bacteria the phoH gene is located in a conserved operon together with diacylglycerol kinase gene (*dgkA*), ybeY homolog gene *yqfG*, and a gene encodes seven-transmembrane-receptor protein with a hydrolase domain (*yqfF*) (Anantharaman and Aravind, 2003).

1.5 A putative Co^{+2}/Mg^{+2} efflux protein YbeX (Escherichia coli CorC)

YbeX is a putative protein also known as *E. coli* CorC protein contain two CBS domains (Feng et al., 2018), putative Co^{+2}/Mg^{+2} efflux protein. YbeX is involved in establishing cobalt resistance and magnesium homeostasis. It is highly conserved among gram negative bacteria present in almost all genomes, that contain phoH gene.

The *ybeX* is located downstream to a well conserved gene *ybeY* among gram negative bacteria. In gram positive bacteria, i.e. *Bacillus subtilis*, *ybeY* homolog *yqfG* is found in an operon where a seven-transmembrane receptor with an intracellular hydrolase domain (7TMR-HD) encoding gene is located adjacent to the YbeY and PhoH genes, and 7TMR-HD is found to be always associated with conserved PhoH-ybeY-diacilglycerol kinase gene neighbhorhood (Anantharaman and Aravind, 2003). Hypoteticaly, a large transmembrane protein (7TMR-HD) in yqfEFG-dgkA operon or a metal eflux protein YbeX in YbeZYX-lnt operon might involve in many membrane associated regulatory process.

Data from a high impact reseach provide an evidence to YbeY to be involved in ribosome biogenesis trough metal regulation. A human made T-ribosome engineered by Mankin and collegues, who tethered two subunits by fusing their ribosomal RNAs into a single molecule, found a nonsense mutation in *ybeX* gene together with missense mutation in ribosomal protein encoding gene rpsA led to a faster growth of *Escherichia coli* SQ171fg/pRibo-T fast growing cells lacking cromosomal rRNA alleles (Orelle et al., 2015). This was later explained by Groisman and collegues, that low cytosolic Mg+2 lead to promoting the importation of Mg+2 where *E. coli* variant with engineered tethered ribosomal subunits faster growth achieved via nonsense mutation in Mg^{+2} efflux protein YbeX (Pontes et al., 2016). In addition, it was conclude that bacteria respond to low Mg^{+2} stress by decreasing ATP levels which enables protein synthesis to proceed because of decreased active ribosomes.

1.6 Inhibition of Escherichia coli growth with Cobalt Chloride

In *Salmonella typhimurium* corB, corC, and corD triple gene deletion mutant enhanced the cobalt resistance (Gibson et al., 1991). In *Pseudomonas stutzeri* depletion of *corC* lead cells to be sensitivity to zinc in RCH2 strain (Vaccaro et al., 2016). The *ybeX* is a putative Mg^{+2} efflux gene and its deletion might cause cells to have increased amount of intracellular Mg^{+2}. It is known that Magnesium cause enhanced Co^{+2} resistance. Mg^{+2} enzymes are known intracellular targets of Co^{+2} toxicity (Foster et al., 2014; Vaccaro et al., 2016).

During inhibition of growth with cobalt chloride, nickel chloride and chloramphenicol the ribosome fractions provides evidence that cobalt involves a specific effect among other tested metal salts on the ribosome (Blundell and Wild, 1969). Protein synthesis had an effect during the inhibition of growth with cobalt while oversynthesis of RNA led to three species of particle accumulation. Later the suggestion was made by Blundell&Wild that cobalt precursors are ribosome precursors so if this is the case the particles should be incorporated into ribosomes when cobalt inhibition revealed (Blundell and Wild, 1973). Indeed, the particles accumulated in E. coli during cobalt inhibition are ribosome precursors and incorpratated into active 70S ribosomes.

2. EXPERIMENTAL PART

2.1 The Aims of the Project

YbeY is a putative ribosomal endoribonuclease which has been implicated, among other things, to be involved in quality control of 70S ribosomes, in 17S pre-rRNA maturation and in ribosomal degradation. However, controversy reigns over its mode of action, substrates, co-factors, and interaction partners.

We attempt to understand the extremely highly conserved protein YbeY in the context of its conserved operon *ybeZYX-lnt*. We experiment on *ybeY* downstream/upstream genes *ybeX* and *ybeZ* deletion mutant strains in *E. coli*, as both genes and their functions unclear.

The aims of this study are

1) To do a comparative study of the phenotypes of *ybeY* and *ybeX* deletion mutant strains in respect to ribosomal metabolism
2) To look for a possible compensatory phenotype of YbeY overexpression

2.2 Material and methods

2.2.1 Bacterial strains, and plasmids

Escherichia coli wild-type (WT) strain from Keio collection, *BW25113*, and deletion strains from the collection were used. Keio collection is a *E. coli* K-12 strain single-gene deletion library. The constructs were made via replacing open-reading frame (ORF) coding regions with kanamycin cassette flanked by FLP recognition target sites (Baba et al., 2006). Strains for gene knockouts of *ybeZYX-lnt* operon were used; *ΔybeZ::Kan*, *ΔybeY::Kan*, *ΔybeX::Kan* (shown also as *ΔybeZ*, *ΔybeY*, and *ΔybeX*). The plasmid that is used to overexpress YbeY protein in *ΔybeX* was constructed in our lab (Hausenberg, 2016) with primer-based cloning, CPEC (Quan and Tian, 2011). Plasmid pET28a kanamycin resistant gene carrying plasmid was used to carry gene *ybeY* from *E. coli* genome to the plasmid.

2.2.2 Dot-Spot experiments

Wild type E. coli and *ybeZYX-lnt* operon deletion mutant strains were used. We set up our experiments in a such way that the cells were grown overnight into stationary phase (Figure

8A). This stationary culture was either directly diluted into fresh LB in 96-well plates for growth curve measurement (Figure 8A, arm 1), or it was first diluted into a 100-ml flask and grown into exponential phase, before dilution into the 96-well plate (Figure 8A arm 2). The cell density was monitored via measuring absorbance at 600 nm and after growth has reached to indicated growth phases, all cultures were diluted to OD_{600} 0.1 and 10 times serial dilutions were made in 96-well plate, and 5µl of each diluted cultures were spotted on LB-Agar or antibiotic containing plates. Plates were incubated at 37°C, except heat shock plates, which were incubated at 42°C or 45°C.

2.2.3 Total RNA Purification

Total RNA was purified with hot phenol-chloroform extraction, as described in (Kasari et al., 2013), with minor modifications. The strains; *Keio WT, ΔybeZ, ΔybeY, ΔybeX* were grown in LB liquid media at 37°C. 10-12 mL of culture were transferred to a Falcon tube and the cells were pelleted with high speed centrifugation (8000 g), frozen in liquid nitrogen and stored at -80°C.

Cells were taken from -80°C and thawed on ice. Cells were taken up with 0,6 mL of 1xTEN buffer (1mM EDTA, 10mM Tris-HCl pH 7, 100mM NaCl) and suspended with up-down pipetting, transferred to a clean 1,5 ml Eppendorf tubes and pre-warmed (65°C); 0,6 mL 1% SDS containing phenol (pH 4.5) was added to the cells and the tubes inverted a few times. The extraction was done at 65°C thermostat for 6-7 minutes and tubes were mixed (end-over-end) once in each minute. Lysates were chilled and the experiment proceed on ice. The samples were centrifuged at 16200 g for 10 min at 4°C to separate phases. The upper RNA containing aqueous phase was transferred to cooled Eppendorf tubes and 580 µL of Chloroform: Phenol (1:1) mixture was added to the collected supernatant. Vortexed vigorously for 10 seconds and centrifuged as above. The supernatant transferred to a clean Eppendorf tube and 500 µL of chloroform added, to get rid of phenol contamination to avoid degradation of the nucleic acid, vortexed vigorously for 10 seconds and centrifuged at 16200 g for 4 min at 4°C. The upper aqueous phase was transferred to a new Eppendorf tube and 0.1 volumes of 3M RNase free sodium acetate (pH 5.2) was added, and RNA precipitated with 1.2 volumes of ice-cold ethanol during incubation at -20°C. RNA was pelleted with centrifugation at 16200 x g 10 min at 4°C. RNA pellets were washed with 1 ml of 70% ethanol. Pellets were air dried on bench for few minutes, and dissolved in ddH_2O and stored at -20°C.

2.2.4 Agarose Gel Electrophoresis

Agarose is weighted and transferred into a clean flask. Appropriate amount of 1xTAE buffer (40 MM Tris-HCl, 20mM Acetic acid, 1 mM EDTA, pH 8.3) is added and the agarose boiled in the microwave oven. The agarose is completely dissolved and cooled down to approximately 60°C. The DNA binding dye ethidium bromide (EtBr) is added (6-8 µL of 10mg/mL stock). The nucleic acid content of the samples was determined via NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific). The samples were diluted in MilliQ water (RNase and DNase free) and equivalent amount of samples were loaded on the gel with 6x DNA loading dye (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA). The gel was replaced into electrophoresis chamber and the separation of the nucleic acid based on size was done via applying voltage to the 1xTAE running buffer.

2.2.5 Ribosome and ribosomal components purification

2.2.5.1 Preparation of crude lysate

E. coli wild type strain and two deletion mutant strains were grown at 37°C in LB liquid media, for deletion mutant strains in the presence of kanamycin (25 µg/mL). Cells were allowed to grow until OD_{600} = 0.3 and then the cell culture were split to two flasks. Chloramphenicol was added to one of the flasks and treated for 2h, the control flask was grown in the absence of antibiotic. The bacteria were then pelleted with low speed centrifugation (centrifuge 4K15, Sigma, 2500 x g, 15 min, at 4°C) and cells suspended in lysis buffer (50 mM Tris-HCl ph 8.0, 60 mM KCl, 60 mM NH_4Cl, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10U/mL DNase I and 3 mg/mL lysosome). The suspension was incubated on ice for 10-15 minutes and then homogenised with French press or bead beater (1g of glass beads d=0.1 mm, BioSpec Products were added to the cells and cells were lysed around room temperature with FastPrep-24 from MP Biomedicals). The cell debris was pelleted by centrifuging the lysate for 50 min at 18 000 rpm in SS 34 Sorvall rotor at 4°C. The A_{260} was monitored and 50U was loaded ot the top of gradients.

2.2.5.2 Sucrose Gradients

10-30% or 10-40% (only Supp. Figure S8) sucrose gradients were prepared via using gradient maker. 30% and 10% of sucrose solution containing 1x LLP (25 mM Tris-HCl pH 7.9, 100

mM KCl, 10 mM $MgCl_2$, 5 mM β-mercaptoetanol) was prepared via dilution from 50% sucrose and 10xLLP buffer. 40-50 unit(U) of (A_{260} 1 is considered as 1U) clarified lysate was loaded on each gradient. The ultracentrifuge tubes were balanced with 1xLLP. The 70S ribosomes and ribosome subunits were separated on sucrose gradients via ultracentrifugation (Beckman Coulter, rotor SW28, 17h, 20400 rpm +4°C, ω^2t=2,8e+11). Samples from gradient were pumped starting from bottom through spectrophotometer (Econo UV Monitor, BIO-RAD, sensitivity 0.5), which can detect different density of different fractions of ribosomal subunits. The results were visualised and recorded via DataAcquisition program. Different fractions (70S,50S,30S) were collected and studied with Northern Blot.

2.2.5.3 Purification of rRNA from Ribonucleoprotein (RNP) Complexes

The ribosomes are composed of RNA called ribosomal RNA (rRNA) and ribosomal proteins. So that the rRNA need to be purified for analysis with northern blotting. Ribosomes and ribosomal subunits were collected from sucrose gradients as a fraction of each peak. Sucrose fractions were collected in 15 mL falcon tubes and diluted at least twice with the gradient buffer 1xLLP. Two and the half volumes of 96% ethanol (EtOH) was added per each sample volume (1V) fraction and kept at -20°C overnight. Following morning the fractions pelleted via centrifugation with 4K15 (Sigma, rotor 11150, 45 minutes 4 krpm, +4°C) centrifuge. The pellet was washed with 70% EtOH and previous centrifugation performed for 10 minutes. The EtOH was poured and the trace was evaporated at room temperature for 5 minutes. The ribonucleoprotein complexes were suspended in 0.1 mL of MilliQ water, and samples were stored at -20°C till rRNA purified.

The rRNA purified with phenol-chloroform extraction as it is described previously. Shortly, the samples were thaw on ice, 60 µL of 10xTEN added, and final volume was taken up to 600 µL with MQ. The samples were kept on ice and 1% SDS containing 600 µL of phenol added to the samples. Samples were vortexed vigorously and kept on ice for 5 minutes, and centrifuged 10 min 13.2 krpm +4°C (centrifuge 5415 R, Eppendorf, rotor F45-24-11). The water phase transferred to a new tube and 580 µL of 1:1 chloroform: phenol mixture was added and vortexed for 10 seconds. The protocol given in section 2.2.3. Followed and RNA precipitated, and stored in MQ at -20°C.

2.2.6 Northern Blotting

Schematic depiction of the Northern blot analysis in general is shown in Annex 2. The method is used from (Kasari et al., 2010; Rio, 2014, 2015) and Sambrook&Russell molecular cloning book 3rd edition (Cold Spring Harbor Laboratory Press, New York).

2.2.6.1 Denaturing Agarose Gel Electrophoresis

The first stage in northern hybridization is separation of RNAs via electrophoresis trough denaturing agarose gels electrophoresis, according to their sizes. The isolated RNA were separated on 1.5% denaturing agarose gel containing 1x MOPS buffer and 2% formaldehyde. The sample and RNA markers preparation holds as described; 3-5 µg of RNA (not more than 6.6 µL in final volume) was mixed with 5.4 µL of formaldehyde, 3 µL of 10x MOPS buffer and 15 µL of formamide. The samples and RNA markers from Thermo Scientific (RiboRuler High Range and Low Range RNA ladder) were denatured at 55°C thermostat for 15 minutes. The RNA mixes were cooled down on ice to prevent secondary structure formation. 3 µL of loading dye bromophenol blue agarose dye (1:6) (0.25%bromophenolblue, 40% sucrose) were added to the samples. And the samples were loaded on the gel in each well and the electrophoresis started. The electrophoresis buffer was the same as the buffer used to prepare the gel, 1 x MOPS. During first hour 60 volt was applied and then the voltage increased to 85 volts.

When the run ended (after 5 hours) the ladder region was cut off with a clean scalpel and stained for 30 min in running buffer containing 10000 times diluted Diamond™ Nucleic acid dye (Promega). In some cases, all the gel was stained instead of proceeding to transferring the RNA to the nylon membrane (Amersham Hyband™- N^+, GE Healthcare).

2.2.6.2 Upward Capillary Transfer of RNA from Denaturing Agarose Gel

The transfer of the RNA from the agarose gel to the nylon membrane was done as described. Capillary transfer of RNA from the denaturing agarose gel to nylon membrane The gel is replaced on the sat up upward capillary transfer Buffer drawn from a reservoir passes through the gel into a stack of paper towels. RNA eluted from the gel by the moving stream of buffer is deposited onto a nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

2.2.6.3 Hybridization of the Northern Blot Membrane

The hybridization buffer and the bottle were heated up at 62°C thermostat in darkness (because the primers used are florescent labelled). 20-25 mL of prewarmed (62 °C) hybridization buffer was transferred to the hybridization bottle, and the membrane was kept rotating in the bottle with buffer for 2h at 62 °C. The florescent labelled DNA-oligonucleotide added in 10 µM final concentration and the hybridization take place overnight.

2.2.6.4 Washing the membrane

The wash buffer (Annex 1) was warmed up in a water bath to 43°C. The membrane was washed with prewarmed (43°C) wash buffer in temperature controlled orbital shaker in a metal box preventing light. The membrane was washed 3 times for 5 minutes with approximately 250 mL of the wash buffer. The membrane was replaced between plastic envelop, and proceed on the detection.

2.2.6.5 Signal Detection and rRNA band quantifications

The scanning of the membrane was done in Amersham Typhoon Gel and Blot Imaging Systems (GE Healthcare). The laser scan is designed for versatile imagining and precise quantification of fluorescent labelled nucleic acids. The quantification of rRNA on the RNA blot images was made via ImageQuant TL (GE Healtcare) image analysis software. The bar plot plotted in R programming where the bars on the plot represent the quantified rRNA bands.

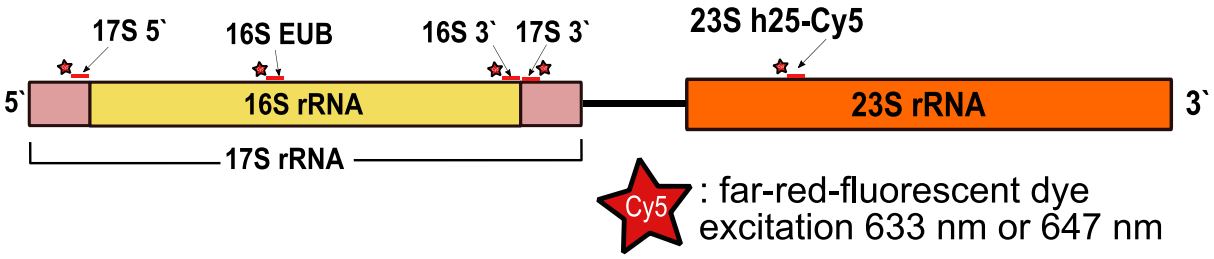


Figure 5 | The Cy5 labelled DNA oligonucleotides and their location on the rRNA. The oligonucleotides were used for Northern blot experiments for specific hybridization and quantification of the RNA species. The table of the oligo sequences are given with their exact location the rRNA in Annex Table A1.

3. RESULTS

YbeY is a 17.5 kDa protein (Zhan et al., 2005) which is claimed to be involved in many cellular processes in bacteria, including rRNA maturation. Deletion of *ybeY* leads to a strong phenotype, including sensitivity to antibiotics and heat, accumulation of aberrant 16S rRNA precursors called 16S* rRNA, and to slow growth (Davies et al., 2010). The mechanistic role of YbeY remains unclear and needs to be studied, although it has been claimed that YbeY is an endoribonuclease acting together with exoribonuclease RNase R to degrade defective ribosomes (Jacob et al., 2013). In many bacteria *ybeY* is located in the *ybeZYX-Int* operon (Anantharaman and Aravind, 2003), where *ybeZ* encodes an ATP binding NTP hydrolase domain containing PhoH subfamily protein (Kim et al., 1993), and *ybeX* encodes a putative Cobalt/Magnesium efflux protein.

We experiment on the deletion strains from Keio collection (Baba et al., 2006). The *ybeZYX-lnt* conserved operon gene deletions, together with isogenic BW11325 strain (WT), were used. A putative Co^{+2}/Mg^{+2} efflux protein YbeX (Escherichia coli CorC) is located downstream to the extremely highly conserved *ybeY* gene. Upstream to the *ybeY* a well conserved gene, *ybeZ,* which is present from *E. coli* to *Bacillus subtilis* (Anantharaman and Aravind, 2003), overlaps by 4 nucleotides with YbeY in *E. coli* and many other bacterial species (Supp Fig …). In some bacteria, i.e. *Rhizobium mesoamericanum STM3625* and *Rhizobium favelukesii*, the *ybeX* is fused with the *ybeY* sequence. Here we conduct different assays with the *ybeZYX-lnt* operon single gene deletion strains in comparison with the WT strain. We present evidence for a functional interaction between *ybeY* and *ybeX*.

3.1 Deletion of *ybeX* in *Escherichia coli* leads to heterogeneous growth

Escherichia coli K12 is one of the most studied and best-characterised organism in molecular biology, making it a useful tool to understand the biology behind genes. Gene disruption is a time-honoured practice in biology that holds the key to understanding the function of a gene. The Keio collection is a constructed systematic *E. coli* K12 single gene mutant library with defined single gene deletions where a specific gene replaced with kanamycin resistance gene via λ red recombination system (Supp. Fig. S3) (Baba et al., 2006; Datsenko and Wanner, 2000).

As the deletions used in this study were not made by us and originate from the Keio collection, we verified the deletion of the *ybeX* gene and the presence of the kanamycin resistance cassette (*kan*) by polymerase chain reaction (PCR) in comparison with wild type *E. coli* strain (Figure

6). The new operon structure, meaning that presence of *kan* resistance gene instead *ybeX*, in *ΔybeX* strain was uncovered via *ybeZ*, *ybeY* and *kan* sequence specific primers. SO30, SO6 and k2 (forward primers) and SO27, SO5, kt and k1 (reverse primers) were used for setting up the PCR reactions (Figure 6A). The presence of kanamycin (*kan*) gene in the ΔybeX strain was tested with primers kt and k2 (Figure 6B line 9, 471-nt fragment). The analysis revealed that while the *ybeX* gene was present in the parental strain BW25113, it had been successfully replaced with *kan* gene in the *ΔybeX* strain.

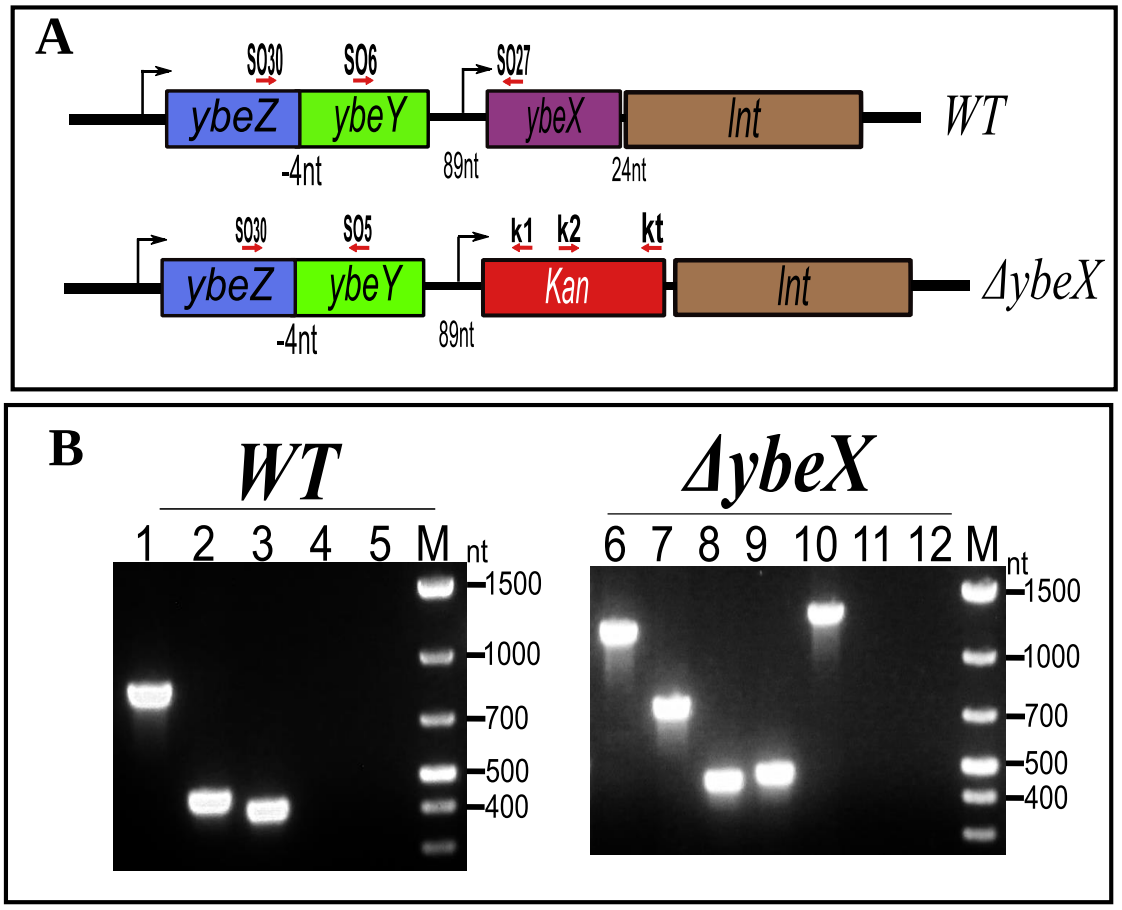


Figure 6. Verification of *ybeX* single gene deletion in *ΔybeX* strain with polymerase chain reaction (PCR). (A) PCR analysis of the *ΔybeX* and WT Keio strains were done with primers (annotated with red arrows on the genes) shown on the ybeZYX-lnt operon. The ybeX gene in *ΔybeX* mutant strain was replaced with kanamycin (*kan*) resistance gene. (B) 1.2% agarose gel electrophoresis of PCR products. Each band on the agarose gel corresponds to a PCR reaction with different sets of forward-revers primers 1-(SO30-SO27), 2-(SO30-SO5), 3-(SO6-SO27), 4-(SO6-k1), 5-(SO30-k1), 6-(SO30-k1), 7-(SO6-k1), 8-(SO30-SO5), 9-(k2-kt), 10-(SO6-kt), 11-(SO30-SO27), 12-(SO6-SO27), 1kb plus DNA marker (M). Negative controls were set for WT (empty lines 4 and 5) via primers aligned with the *kan* resistance gene. The primer SO27 don`t have the specific sequence to bind in *ΔybeX* (empty lines 11 and 12).

Deletion of *ybeY* causes a growth defect in *E. coli*, so the colonies formed on LB Agar plates are smaller than wild type. In contrast, deletion of *ybeX* leads to a variety of colony sizes, when the cells are plated directly from a glycerol stock (Figure 7). Re-streaking small colonies and large colonies from these plates invariably results in uniformly large 2nd generation colonies (data not present), showing that small colonies are not genetically determined. This result suggests that deletion of *ybeX* leads to a complex phenotype that depends on the initial physiological state of the cells. Such phenotypic heterogeneity could conceivably arise from the shock of making or freeze-thawing the glycerol stocks, or from growth in the liquid medium prior to adding the glycerol.

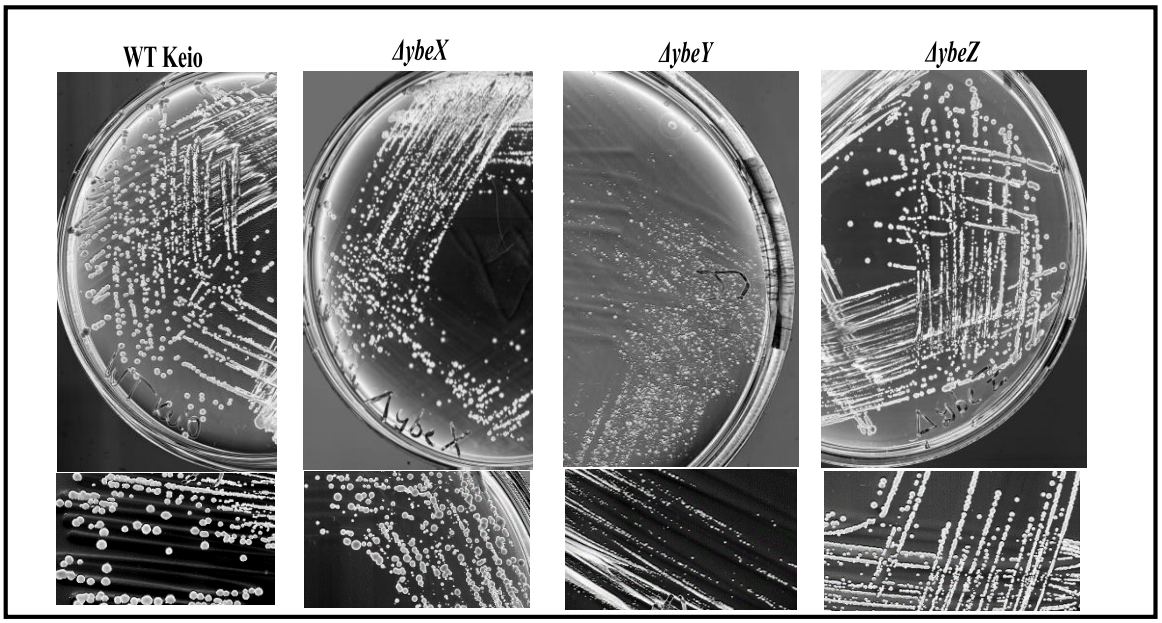


Figure 7 | Colony growth of wild type, *ΔybeZ*, *ΔybeY*, and *ΔybeX E. coli* strains from Keio collection on LB agar plates. The kanamycin resistance gene which was replaced with the deleted gene is present in *ΔybeZ*, *ΔybeY*, and *ΔybeX E. coli* strains. The bacteria were taken from Keio glycerol stocks which is stored at -85°C, streaked onto LB-Agar plates, and incubated at 37°C overnight (14-16 hours). The whole plates with colonies are shown together with the lower zoomed in pictures.

To test the suggested possible scenarios for such heterogeneity and glycerol stock effect, we grew *ΔybeX* and WT *E. coli* strains at 37°C in LB liquid media, and streaked on LB plates directly from the overnight cultures. The same cultures were used to prepared glycerol stocks, and cells were streaked on LB plates from. We observed the same heterogeneity of colony sizes on *ybeX* plates, but not on WT plates, regardless of whether glycerol stocks were made of the stationary phase cultures (Supp. Fig. S2). This result indicates that stationary phase *ΔybeX* cells

have heterogeneous physiological states, which lead to heterogeneous growth resumption and/or growth rates.

3.2 ΔybeX cells exhibit slow outgrowth from the stationary phase, resulting in a prolonged lag phase

When stationary phase bacteria are transferred into fresh medium, the cells exhibit a growth phase called lag phase with no growth, which is the first and most poorly understood stage of bacterial growth (Madar et al., 2013). Although during lag phase bacterial biomass does not increase, lag phase seems to prepare bacteria for exponential growth, and it involves transient metal accumulation (Rolfe et al., 2012). We encounter a longer lag time for *ybeX* deletion mutant in comparison to wild type E. coli strain when cells are diluted into fresh media.

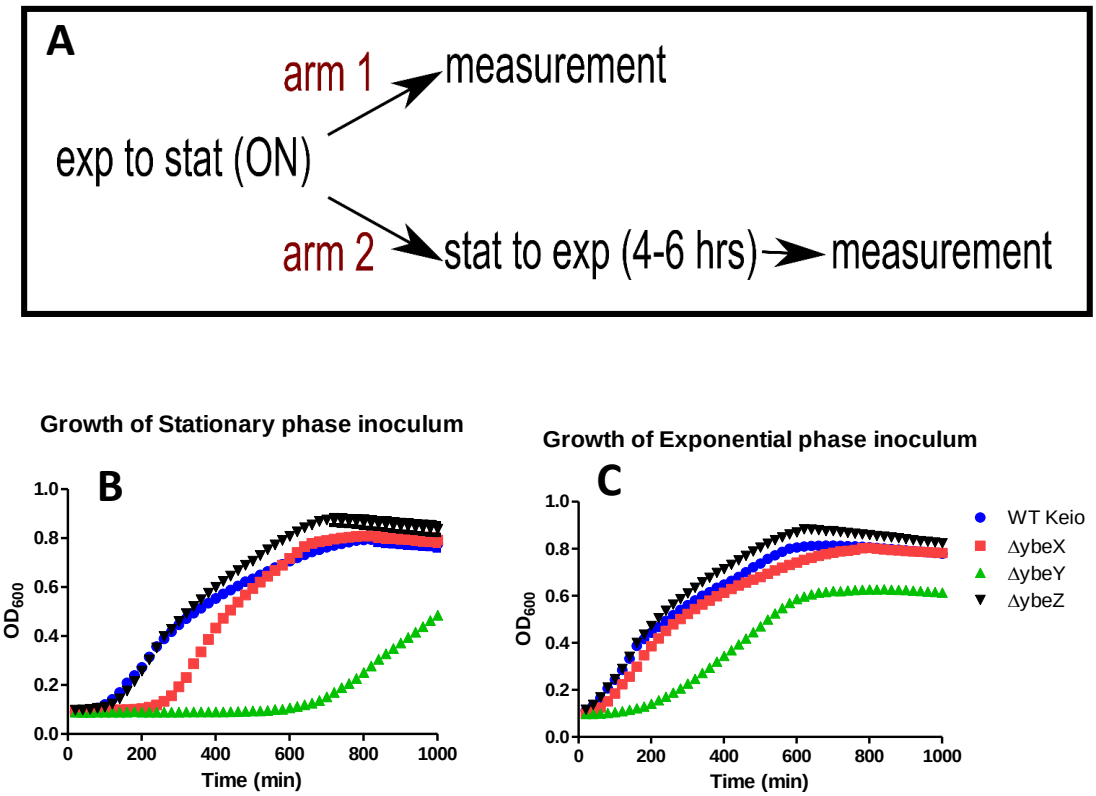


Figure 8 | (A) A scheme for the experimental set up. In the first arm of the experiment the cells were grown over-night (ON) in 3 ml minipreps from exponential phase (exp) to stationary phase (stat). The growth measurement either involves diluting cultures into fresh LB in 96-well plates (shown in panel B, C), or making serial dilutions onto LB-agar plates (Figures 9-10). In the second arm the stationary cells were, before measurement, diluted into liquid LB medium, grown there back into exponential phase. Arrows denote dilutions of cultures. (B) The growth curves in liquid media in the arm 1 of the experiment (C) The growth curves in the arm 2 of the experiment. Blue curves represent the growth of WT bacteria, red curves represent the ybeX cells, green curves the ybeY cells and black curves the ybeZ cells. x- axis shows the time in hours and y- axis show the monitored cell density at OD_{600}.

We set up our experiments in a such way that the cells were grown overnight into stationary phase (Figure 8A). This stationary culture was either directly diluted into fresh LB in 96-well plates for growth curve measurement (arm 1), or it was first diluted into a 100-ml flask and grown there into exponential phase, before dilution into the 96-well plate (arm 2). All arm 1 cultures consistently exhibited longer lag phases than the corresponding arm 2 cultures (Figure 8 B-C). Interestingly, in arm 1 (stationary phase inoculum), but not in arm 2 (exponential phase inoculum), the Δ*ybeX* cells exhibit a prolonged lag phase in relation to WT and *Δybe*Z cells. This indicates that the physiological heterogeneity of *ΔybeX* seen at the cellular level (Figure 7) translates into a longer lag phase at the aggregate level (Figure 8B).

3.3 YbeX Is a Heat Shock Protein

It has been shown that *ybeY* deletion mutants in different bacterial species have significantly reduced thermotolerance (Pandey et al., 2011; Vercruysse et al., 2014; Warner, 2013). We tested *ΔybeX* at heat shock temperatures (42°C and 45°C) in a dot spot assay in comparison with *ΔybeY*, *ΔybeZ*, and the isogenic wild type strain. The experimental setup was essentially the same as depicted in Figure 8A (the drawn figure see supp. Fig. S1), with the difference that cell growth was not measured in 96-well plates, but instead by plating out series of dilutions of the cultures onto LB-agar plates. These agar plates were incubated at 37°C, 42°C and 45°C, as indicated in Figure 9.

We discovered that the deletion of *ybeX* has a similar heat shock phenotype to *ΔybeY*, albeit this phenotype only occurs when cells enter the stationary phase (Figure 9). The *ybeX* mutant cells collected from stationary growth phase already have a growth defect at 37°C, and this effect becomes much more pronounced at higher temperatures. In contrast, *ybeX* cells collected from exponentially growing liquid cultures do not exhibit a growth phenotype at any temperature tested by us (see the lower row labelled “Exponential” in Figuren9).

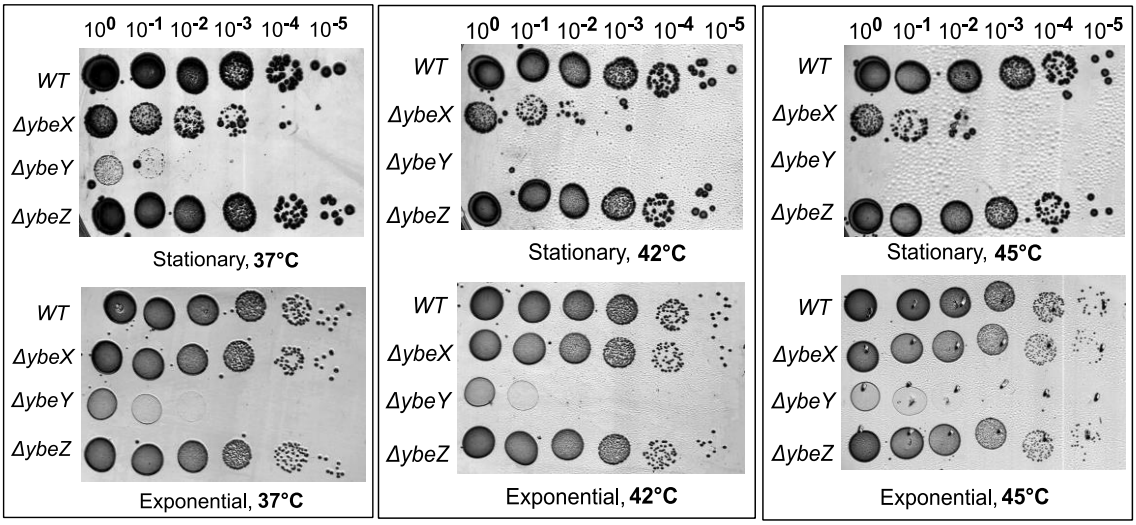


Figure 9 | Temperature dependent growth assay, or dot-spot experiment, for *Escherichia coli* Keio *wild type* (*WT*) strain and *ybeZYX-lnt* operon gene deletion mutants at 37°C, 42°C and 45°C. Deletion of *ybeX* in *E. coli* and its effect to the cell survival is shown in comparison with wild type, *ΔybeY*, and *ΔybeZ*. The dots on the LB agar plates represent a serially diluted cell culture from Stationary or Exponential states, as indicated in Figure 8A. The strains were grown overnight in LB liquid media and from these overnight cultures (considered stationary phase) 100x dilution were made into fresh LB liquid media and grown untill OD_{600} reached 0.5-0.7 (considered exponentially growing cells). 10 times serial dilutions (indicated on top of the panels) were made and 5µL of each dilution was spotted onto LB-Agar plates and incubated at indicated temperatures.

Deletion of ybeZ in E. coli showed no phenotype, behaving similarly to wild type on all plates. *ΔybeY* plated from stationary cultures led to no colonies on heat shock plates, while there are some colonies at 37°C. In contrast, the *ΔybeY* cultures plated from exponentially growing cultures led to similar numbers of colonies in all plates. This behaviour is qualitatively similar to that of the *ΔybeX* strain, suggesting a biological connection between YbeY and YbeX proteins.

3.4 *ΔybeX* cells are sensitive to Chloramphenicol and Erythromycin

It was already shown that *ybeX* depletion cause antibiotic sensitivity (Smith et al., 2007; Suzuki et al., 2017). In addition, considering that deletion of ybeX causes defects in the cells, we decided to perform additional dot spot experiments to test the sensitivity of ΔybeX cells towards antibiotics. The experiment was done as described above, except cells were spotted on LB agar plates containing antibiotics at low concentrations (sub-inhibitory for WT): chloramphenicol, erythromycin, rifampicin, and tetracycline (Figure 10, Supp. Fig. S7). As the

ybeY deletion has an extreme growth phenotype even in the absence of antibiotics, the results of adding antibiotics to this strain cannot properly be interpreted. We found that deletion of ybeX leads to profound sensitivity towards the 50S ribosomal subunit-affecting antibiotics erythromycin and chloramphenicol, but only in arm 1 of the experiment, where cells are plated directly from stationary phase cultures. In arm 2 (where exponentially growing cells are plated) ΔybeX exhibits no sensitivity to antibiotics at concentrations tested. ΔybeZ cultures have no sensitivity to erythromycin, but, intriguingly, exhibit equal sensitivity to chloramphenicol in both arm 1 and arm 2 of the experiment. Neither strain exhibits extra sensitivity to 30S subunit-affecting antibiotic tetracycline (Supp. Fig S7). This is unexpected, as we expect the YbeY system to be heavily involved in small ribosomal subunit processing and assembly (see Literature Review).

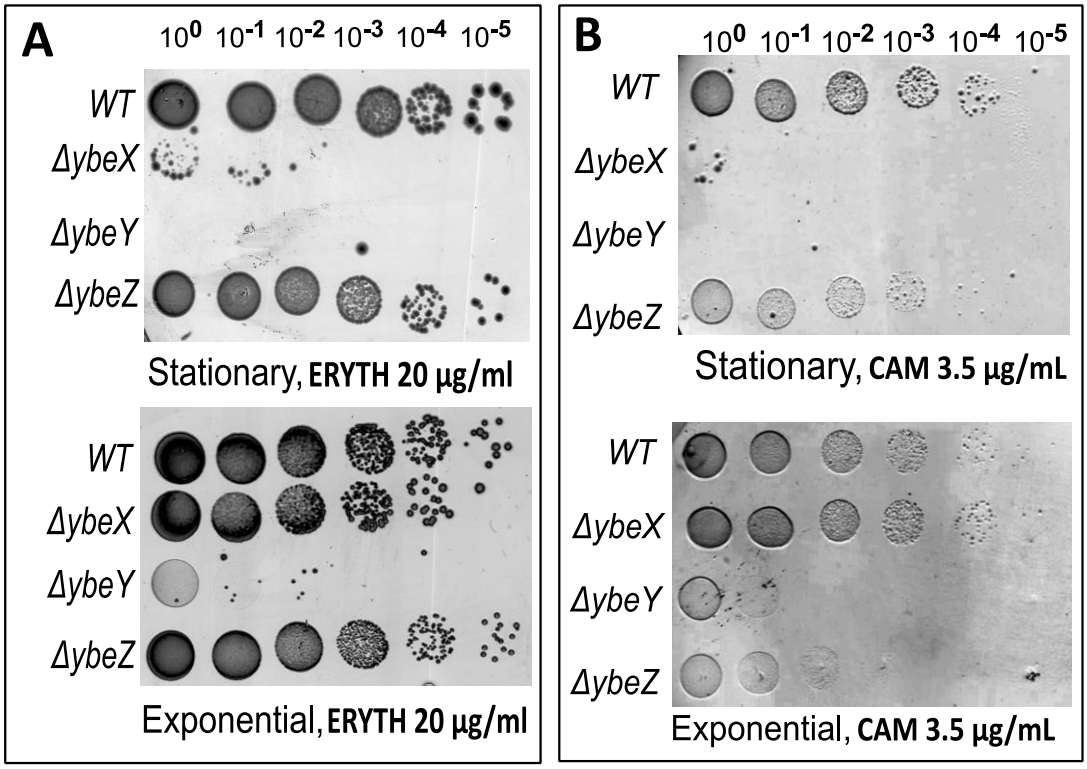


Figure 10 | Dot-spot experiment of antibiotic sensitivity. Wild type (WT) *E. coli* and *ybeZYX-lnt* operon gene deletion mutants at (A) 20 µg/mL erithromycin (ERYTH) and (B) 3.5 µg/mL chloramphenicol (CAM) containig plates. The experiment was done as described in Figure 9. Wild type and deletion mutant strains were grown in LB liquid media and cells were collected at indicated growth stages. Serially diluted cell cultures were dotted on the LB-Agar plates containing antibiotics and the plates were incubated at 37°C overnight.

The transcription-inhibiting antibiotic rifampicin also did not bring out any extra sensitivity in dot spot experiments for Δ*ybeX* and Δ*ybeZ* cells in comparison to isogenic wild type (Supp. Fig. S7). We also determined the minimal inhibitory concentrations (MIC) on our strains (Table 2).

Antibiotics/Strains	***Wt Keio***	***ΔybeX***	***ΔybeY***	***ΔybeZ***
Chloramphenicol (CAM)	15	10	2.5	15
Rifampicin (Rif)	150	80	20	150
Erythromycin (ERYTH)	10	5	0.625	5

Table 2 | The minimal inhibitory concentrations (MICs, mg/L) of wild type *E. coli, ΔybeX, ΔybeY,* and *ΔybeZ* strains. Lowest concentration of antibiotics that inhibits the visible growth after overnight incubation for 4 *E. coli* strains are given in mg/L units.

3.5 The ΔybeX mutant is defective in rRNA maturation

The ribosome is composed mainly of rRNA (Wilson and Nierhaus, 2007) and mistakes in processing of rRNA can lead to defects in the ribosome and its function (Wireman and Sypherd, 1974) that affect the translation of genetic information into functional proteins. YbeY was claimed to be involved in two crucial physiological functions; (1) in late-stage 70S ribosome quality control that is particularly important under stress, and (2) in processing of the 16S rRNA 3`end (Jacob et al., 2013). Here we look at the processing of rRNA in *ΔybeX* strain in comparison with the wild type and the *ΔybeY* strains. We purified total RNA from wild type and the three deletion mutants *ΔybeZ* , *ΔybeX*, and *ΔybeY*. The comparison is shown only for two deletion strains, *ΔybeX*, and *ΔybeY*, because *ΔybeZ* did not exhibit any defect in rRNA maturation, always behaving similarly to wild type (data not shown).

The total RNA composition was compared from cells collected at different growth stages at 37°C and under heat stress (45°C) in Figure 11. The *ΔybeY* mutant showed, as was seen previously (Davies et al., 2010), accumulation of 16S rRNA precursor (17S rRNA) and of a faster migrating species (16S* rRNA), indicating a defect in 16S rRNA maturation.17S rRNA is present in all stages of growth in the *ΔybeY* mutant. Deletion of *ybeY* causes a complete loss of mature 16S rRNA when cells are kept at 45°C for 1 hour, which is consistent with the fact that ybeY is essential at heat shock temperatures (Jacob et al., 2013) (Figure 11 time point 4).

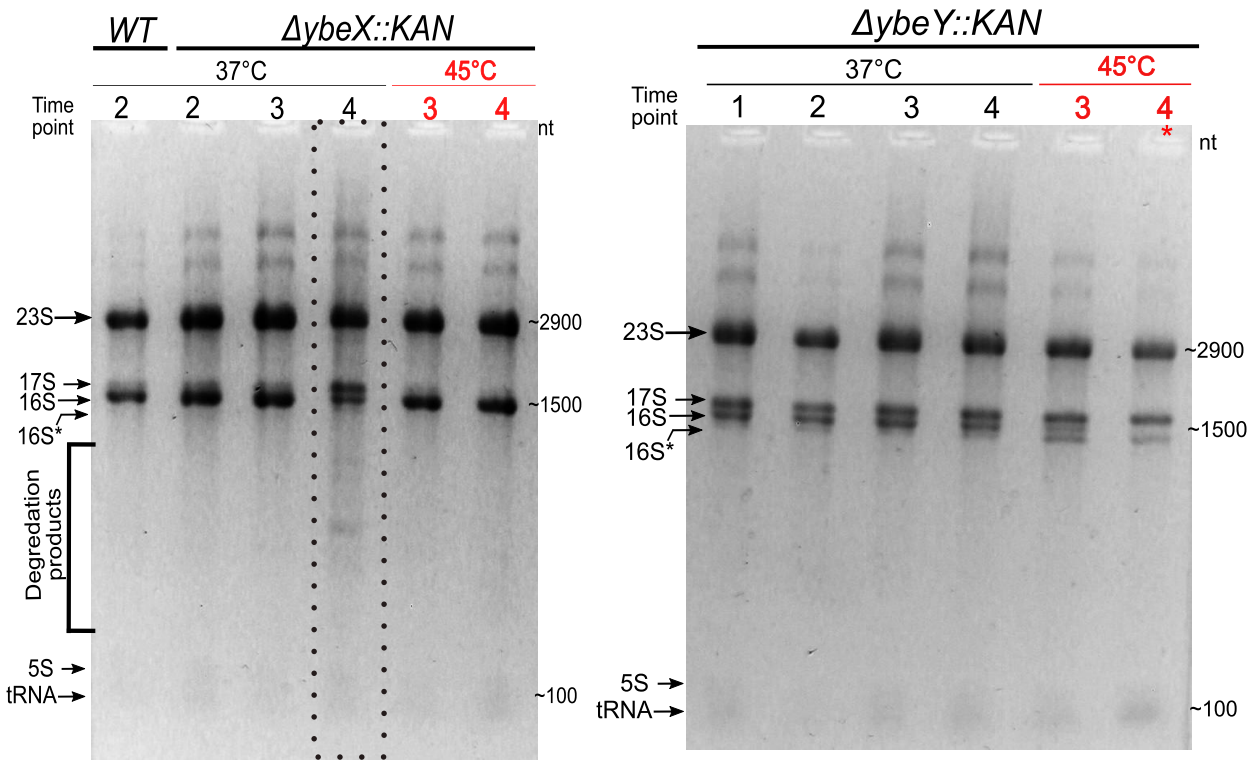


Figure 11 | 1.8% Agarose gel electrophoresis of total RNA from *ΔybeY*, *ΔybeX* and wild type (WT) *E. coli* strains. The cells were grown in LB liquid media at 37°C or 45°C shaker and cell density was monitored via measuring OD_{600} units. Indicated time points are: 1 - OD_{600} = 0.2; 2 - OD_{600} = 0.3; 3 - cells were grown for additional 30 minutes at indicated temperatures (from the 2. Time point); 4 - cells were grown for a total of 1h (from the 2. Time point). The time points in red indicate cells under heat stress at 45°C. The total RNA was purified with hot phenol extraction. The positions of 23S, 17S, 16S and 16S* rRNAs are indicated based on their mobility on the agarose gel. The black box indicates the only time point where heterogeneous degradation products together with accumulated 17S rRNA can be observed (also marked as "Degradation products" at the long edge of gel) in *ΔybeX* cells. Purified wild type *E. coli* total RNA was loaded as a mobility control for 16S, 17S and 16S* rRNAs.

The *ΔybeX* strain showed the presence of a substantial amount of 17S rRNA on 1.8% agarose gel when the cells were grown for additional one hour after cell density has been reached 0.3 (Figure 11). On the other hand, when the *ΔybeX* cells were grown under heat stress (at 45°C for 1 hour), the rRNA profile stayed the same (unlike in *ΔybeY* cells).

The comparison of rRNA profiles on agarose gel electrophoresis between *ΔybeX* and *ΔybeY* shows that deletion of *ybeX* causes an effect at 37°C when the cells are grown in LB liquid media, but not at 45°C. Although the *ΔybeY* cells showed no accumulation of degradation products, *ΔybeX cells* do accumulate aberrant rRNA species.

3.6 Depletion of *ybeX* leads to decreased amounts of 16S and 23S rRNAs

Denaturing agarose gel was used to achieve a better separation of the RNA species than in regular agarose gels. 5 µg of total RNA was used (Figure 12). The rRNA composition of *ΔybeX* cells seems to be changing over the time course of the experiment (Figure 12). As the *ΔybeX* cells near the stationary phase their relative proportion of 16S rRNA is reduced (Figure 12 see lane 12) and that of 17S appears to be increasing (lanes 6-8).

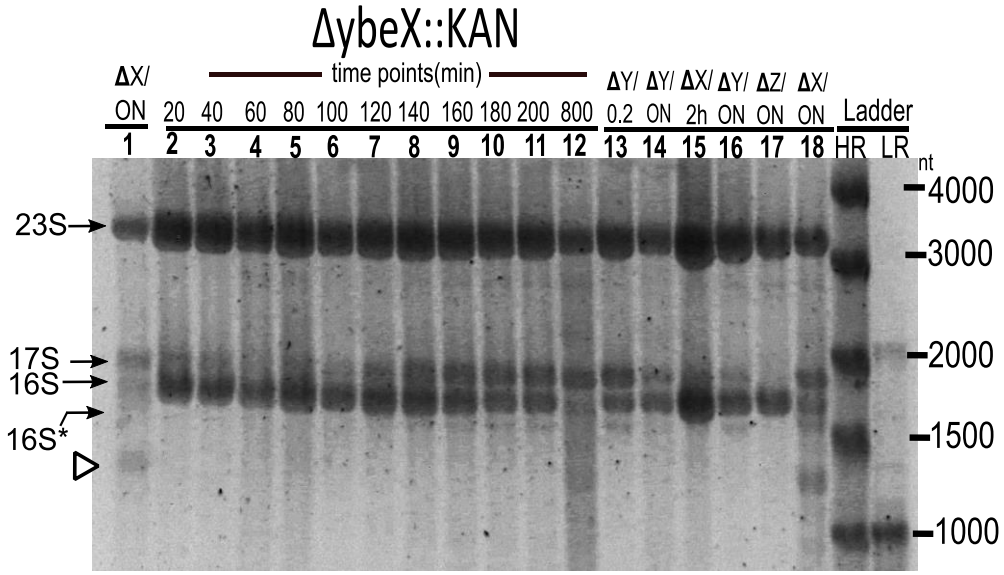


Figure 12 | Denaturing 1.5% agarose gel electrohoresis of total RNA purified from ΔybeX, ΔybeY and ΔybeZ E. coli strains. The strains were shortly shown; *ΔY ~ ΔybeY*, *ΔX ~ ΔybeX,* and ΔZ ~ ΔybeZ *E. coli* strains. Line 13 annotate the ΔybeY strain grown till OD_{600} 0.2. In line number 1-14-16-17-18 cells were grown overnight (indicated as ON). The positions of 23S, 17S, 16S and 16S* rRNAs are indicated based on their mobility on the agarose gel. The distinct accumulated rRNA species are annotated with arrow (▷). High range (HR) and low range (LR) riboruler RNA markers are not well representative of the RNA sizes due to unequal amount of loaded samples and electrophoresis shift.

The longer lag phase which was observed for *ΔybeX* "Stationary" cells inoculated into fresh media (Figure 8B) might cause of accumulated 17S rRNA and degradation products. Overnight grown *ΔybeX* cells accumulated rRNA species are disappeared in cells start to grow (compare Figure 12 lane 1 and 14 with lane 2-4).

3.7 ΔybeX cells accumulate 17S rRNA during growth

To better assess the dynamics of rRNA over the growth curve, Northern hybridization techniques were employed. Northern hybridization allows to measure the amounts and sizes of RNAs. The oligonucleotides used for hybridization allow for high sensitivity of detection and for high selectivity. Determination of the rRNA species was done using specific oligonucleotides indicated in the Materials and Methods section. The *ΔybeX* strain was grown

in LB liquid media into exponential growth phase (OD_{600} = 0.15), and the cells were collected at 20 minute intervals. The total RNA was purified for northern blotting. Each lane contains the same amount of total RNA, most of which is not in the 17S pre-rRNA form and is invisible in the gel. However, this setup ensures a quantitative picture of 17S pre-rRNA accumulation as a fraction of total RNA.

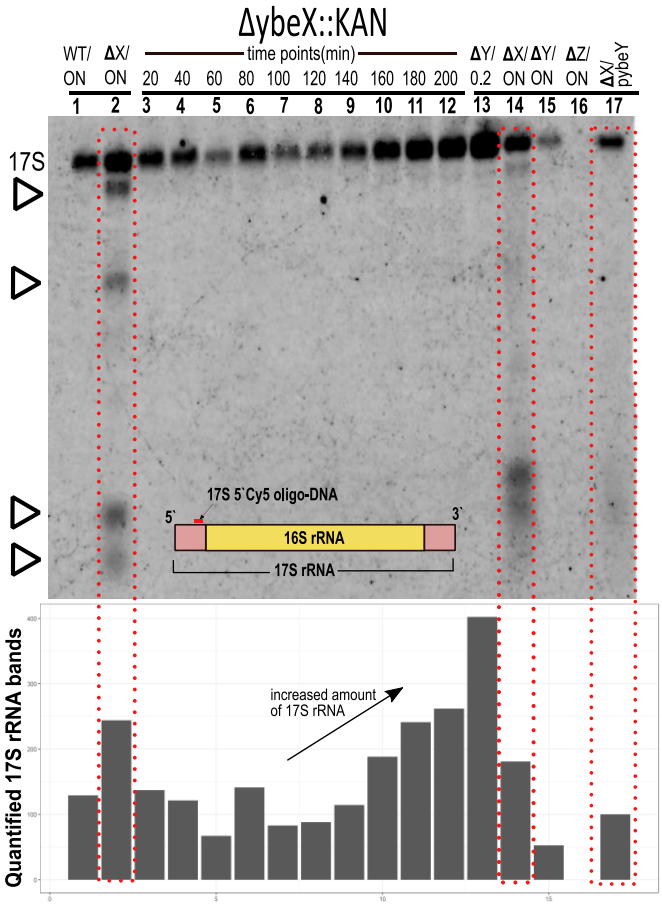


**Figure 13 | Northern blot analysis of purified total RNA with 17S 5`-end specific primer and its quantification.** 17S 5`-end specific DNA-oligo was used for the membrane hybridization (the oligo location on 17S rRNA is shown, and will always be shown for Northern hybridization). This oligo does not bind to mature fully processed 16S rRNA. The bars represent the quantified 17S rRNA. X-axis shows the samples in the same order as on the gel image, and y-axis shows the relative intensity of quantified bands. In marked area, line 2-14-17, the effect of overexpression of YbeY (with plasmid *ybeY* annotated as pybeY, line 17) on *ybeX* deletion mutant strain is shown in comparison with RNA purified from two independent overnight grown *ΔybeX* culture. The arrow on the bar plot annotate dramatic increase in the amount of 17S rRNA when cells are grown in LB liquid media over the time. The strains were shortly shown; WT ~ wild type *E. coli* strain, *ΔY ~ ΔybeY*, *ΔX ~ ΔybeX*, and ΔZ ~ *ΔybeZ*. Line 13 annotate the *ΔybeY* strain grown till OD_{600} 0.2 and cells were harvested and RNA purified. Line 1-2-14-15-16-17 cells were grown overnight. The RNA content of plasmid YbeY transferred in *ΔybeX* strain (*ΔX /pybeY*) and overexpressed, shown on the line 17.

We observed an interesting pattern of 17S rRNA accumulation in ybeX cells wherein a reduction of 17S pre-rRNA in early to mid-exponential phase is followed by a renewed accumulation of this RNA species as the cells gradually approach stationary growth phase (Figure 13 lanes 3-12). This suggests that the accumulation of the unprocessed rRNA species in the *ΔybeX* strain could be most pronounced in the stationary phase itself. This is indeed what was observed in two independent experiments, where total RNA was purified directly from *ΔybeX* cultures (Figure 13 lanes 2, 14), in both of which an apparently full-length 17S pre-rRNA species and several distinct degradation products are observed. Note that the accumulating degradation products, while distinct, are quite different in the two replication experiments. Moreover, overexpressing the YbeY protein from plasmid in the *ΔybeX* background leads to disappearance of those stationary phase specific 17S pre-rRNA derived degradation products (lane 17). Overexpression of YbeY in *ΔybeX* cells also seems to decrease the amount of full-length 17S rRNA. There is also quite a bit of the 17S pre-rRNA in WT cells collected from stationary phase cultures (lane 1), but very little or not at all in corresponding ybeY and ybeZ cultures (lanes 15, 16), suggesting varying physiological responses, and therefore varying conditions in seemingly straightforward replications of stationary phase growth in liquid LB medium. This problem clearly requires further experimental action.

Although evidence has been presented that *ybeZ* and *ybeY* are interaction partners (Vercruysse et al., 2016), deletion of *ybeZ* in *E. coli* has no effect on rRNA profile (Figure … line 16).

3.8 Partial rescue of the ybeX phenotype by overexpressing the YbeY protein

We hypothesized that YbeX and YbeY might be involved together within the same pathway to accomplish ribosomal biogenesis. Therefore, we overexpressed YbeY in exponentially growing ΔybeX strain. *ybeY* gene was constructed in pET28a (under T7 promotor, kanamycin resistance) and 10 mM IPTG was used for induction. The Northern blot analysis reveal that over-expression of the metalloprotein YbeY in *ΔybeX* background decreased the amount of 17S rRNA and increased the amount of mature 16S and 23S rRNA (Figure 14-15).

On the other hand, overexpression of the YbeY protein did not rescue the phenotype of accumulating rRNA degradation intermediates in stationary phase (Figure 14 lane 17). The experiments of overexpressing YbeX protein in *ybeY* deletion background are currently in the works.

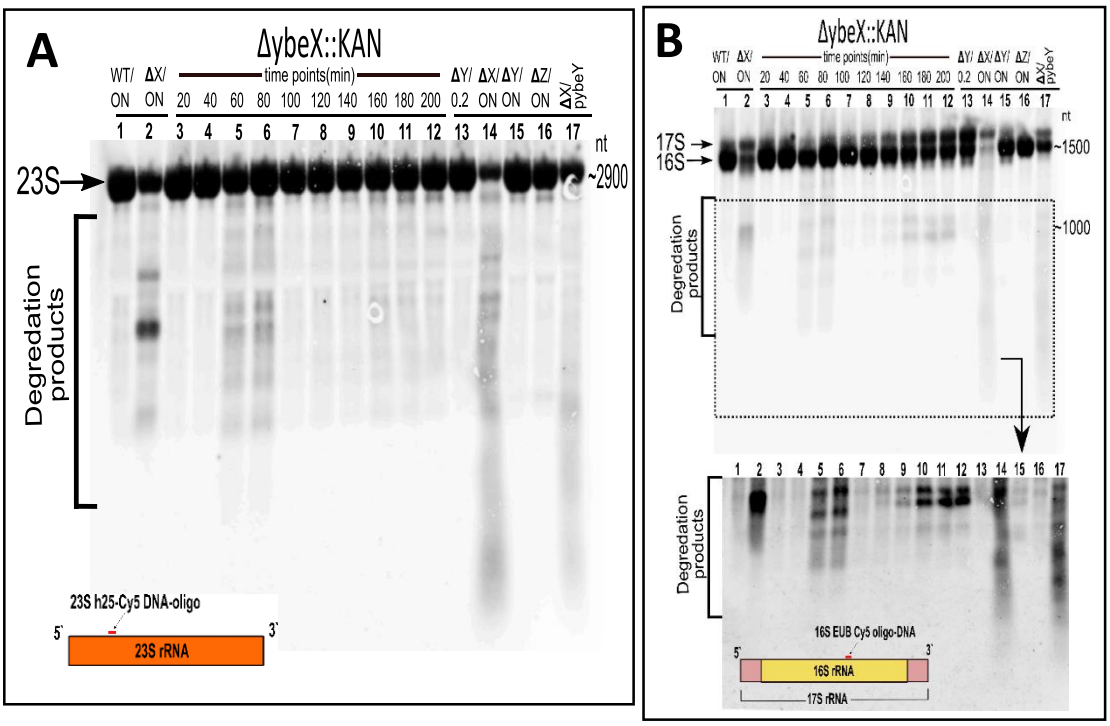


Figure 14 | Northern blot analysis of purified total RNA. (A) 23S helix 25 region specific oligo was used. The oligo location on 23S rRNA is shown. This oligo does not bind 16S rRNA. Line 2-14 compare to line 17, the effect of overexpression of YbeY (with plasmid *ybeY* annotated as pybeY, line 17) on *ybeX* deletion mutant strain is shown in comparison with RNA purified from two independent overnight grown *ΔybeX* culture (lane 2 and 14). The strains were shortly shown; WT ~ *wild* type *E. coli* strain, *ΔY ~ ΔybeY*, *ΔX ~ ΔybeX*, and ΔZ ~ ΔybeZ. Line 13 annotate the ΔybeY strain grown till OD_{600} 0.2 and cells were harvested and RNA purified. Line 1-2-14-15-16-17 cells were grown overnight. The RNA content of plasmid ybeY transferred in *ΔybeX* strain (*ΔX /pybeY*) and overexpressed, shown on the line 17. (B) 16S rRNA specific DNA-oligo was used for the membrane hybridization (the oligo location on 16S rRNA is shown). The oligo binds to 16S rRNA from 338-356 nt.

Figure 13 and Figure 14 originates from the same membrane which was hybridize with different oligo`s. The oligonucleotides with a specific binding regions on the 16S,17S or 23S rRNA binding sites on the rRNA is shown on the figure and also in materials and methods. Decrease in mature 23S was observed in YbeX depleted cells (Figure 14A lanes 2, 14). Deletion of *ybeX* led cells to accumulate distinct 23S rRNA species in two independent experiments, where total RNA was purified directly from *ΔybeX* cultures (Figure 14A lanes 2,

14), in both of which an apparently full-length 17S pre-rRNA species and several distinct degradation products are observed. Note that the accumulating degradation products, while distinct, are quite different in the two replication experiments.

3.9 Depletion of *ybeY* lead to loss of 70S ribosomes at 40°C

Depletion of YbeY in E. coli leads to a major shift from polysomes to single ribosomes and free ribosomal subunits (Rasouly et al., 2009). We did gradient ultracentrifugation experiments using 10-30% sucrose gradients, so as to be focused on the 70S ribosomes and on the free ribosomal subunits, while ignoring the polysomes.

We grew *ΔybeY* cultures in LB media and harvested cells at different 600 nm optical density readings. *ΔybeY* strain was grown in 2L LB liquide media and cells were harvested when OD_{600} 0.2. The bacteria lyzed with french press, and the lysate clarified with high speed centrifugation which was later loaded on the top of prepared sucrose gradients (Supp. Fig S9A). Furthermore *ΔybeY* strain was grown until OD_{600} 0.5±0.1, the bacteria harvested, lysed, the lysate clarified and loaded onto sucrose gradients (Supp. Fig S9B). *ΔybeY* starin was gromw until OD_{600} 0.3 and additional 2h growth was applied and then cells were harvested. The bacteria lysed, the lysate clarified and loaded onto sucrose gradients (Supp. Fig S9C).

There is a dramatic decrease of 70S ribosomes in *ΔybeY* cells grown in LB liquide media at 37°C (Supp. Fig. S9). When cultures were shifted to 40°C for 1 hour to induce heat stress, the 70S peak completely dissapears(Supp. Fig. S8).. This shows that depletion of the YbeY leads to greatly reduced thermotolerance of *E. coli*.

3.10 Chloramphenicol treated ΔybeX cells accumulate abnormal particles

It has been shown that cells incubated with protein synthesis inhibitors, such as chloramphenicol, accumulate aberrant particles whose sedimentation is slower than mature ribosomal subunits (Kurland et al., 1962). Chloramphenicol (Cam) is an inhibitor of the large ribosomal subunit and it affects the assembly of both the large and the small subunit (Siibak et al., 2009).

Wild type E. coli, *ΔybeY*, and *ΔybeX* strains were grown in LB liquid media and cell density was monitored. When cell density reached 0.3 units, the cultures were splitted into two flasks,

one of which was treathed with chloramphenicol (CAM, 7 µg/mL) for 2 hours. The flasks with no antibiotics were grown under the same conditions, at 37°C shaker.The cells were harvested, suspended in ice-cold buffer and homoginized with high pressure homoganizator. The lysate was clarified with high speed centrifugation and 50U of (260 nm absorbance unit) the clarified lysate was loaded onto sucrose gradients and ultracentrifuged (17400 rpm, 17h, SW28 rotor).

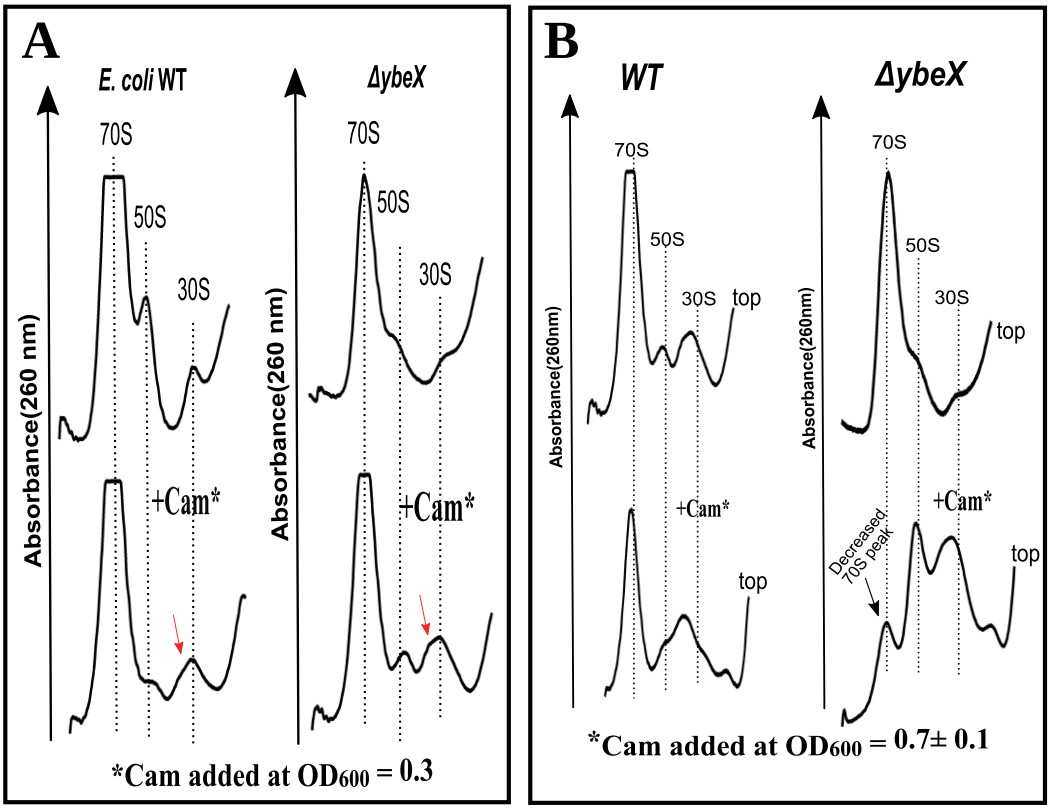


Figure 15 | 10-30% Sucrose density gradient centrifigation profiles from E. coli wild-type and *ybeX* deletion mutant strain (ΔybeX). (A) BW25113 wild type and ΔybeX E. coli strains were grown in LB liquid media at 37°C and the OD_{600} was monitored. When cell density reached 0.3 units, the cultures were splitted into two flasks, one of which was treathed with chloramphenicol (CAM, 7 µg/mL) for 2 hours. The flasks with no antibiotics were grown under the same conditions. **(B)** Bacteria were grown in LB liquid media and the chloramphenicol treatment started when cell density at OD_{600} = 0.7 for both strains. The antibiotic treatment last for 2 hours while the control growth with no antibiotic were grown under the same conditions too. The chloramphenicol effect on the sedimentation profiles shown with (+Cam*) below in comparison with no treated cells. Cells were lysed with high pressure homogenization (French press) and lysate clarified with centrifugation. 50U (A260 = 1 is defined as a one unit) of the lysate was loaded on the top of the prepared sucrose gradients of each. Lower panel gradients are with chloramphenicol treatment while control experiment gradients (no treatment) are in the upper panel. Red arrows point to abnormal ribosomal particles.

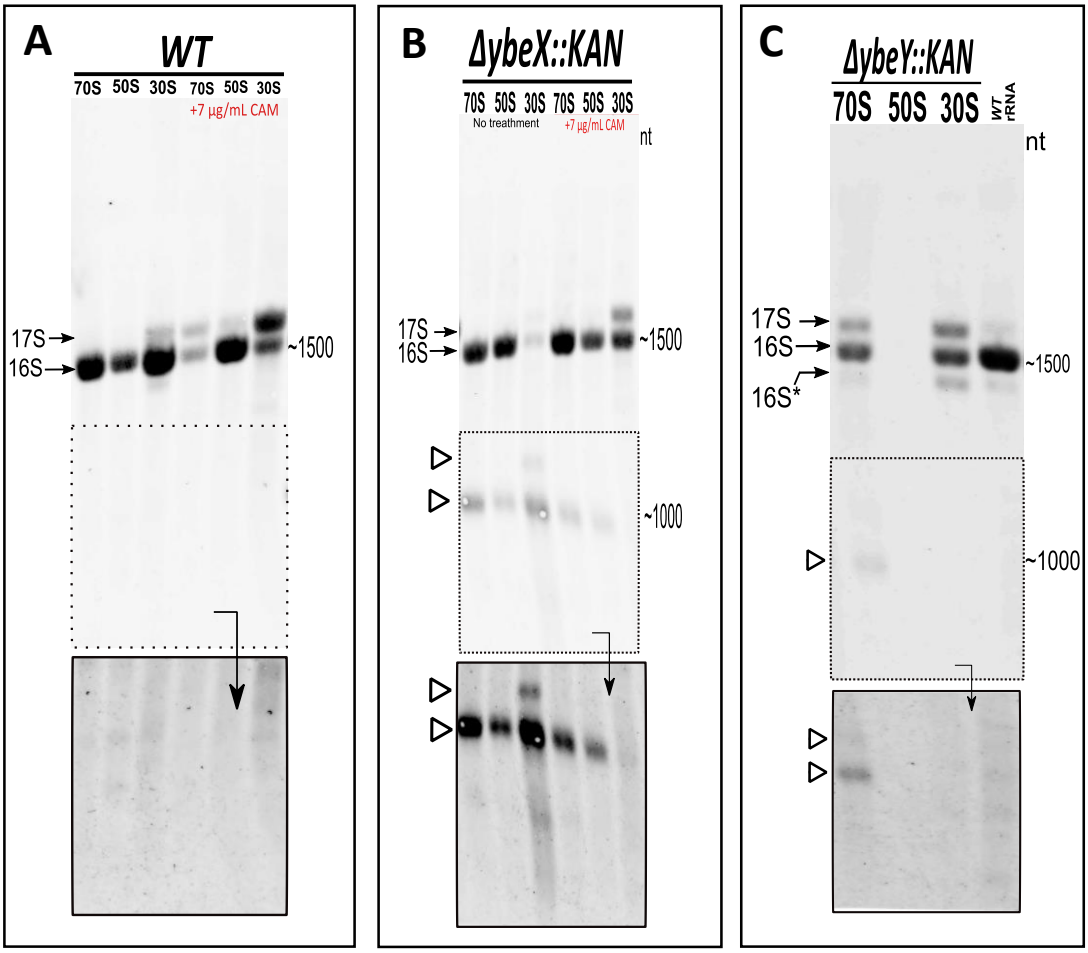
Our group has previously shown that treatment with CAM (7 µg/mL) induced aberrant ribosomal particles in WT and in ΔybeY strains overexpressing YbeY from a plasmid, while the CAM treatment did not induce any extra particles in *ΔybeY* strain (supp. Fig…). Nevertheless, we do observe aberrant ribosomal particles in the *ΔybeX* strain after CAM-treatment, very similarly to the wild type strain (Figure 15A). The accumulated assembly intermediates are indicated with red arrows in Figure 15.

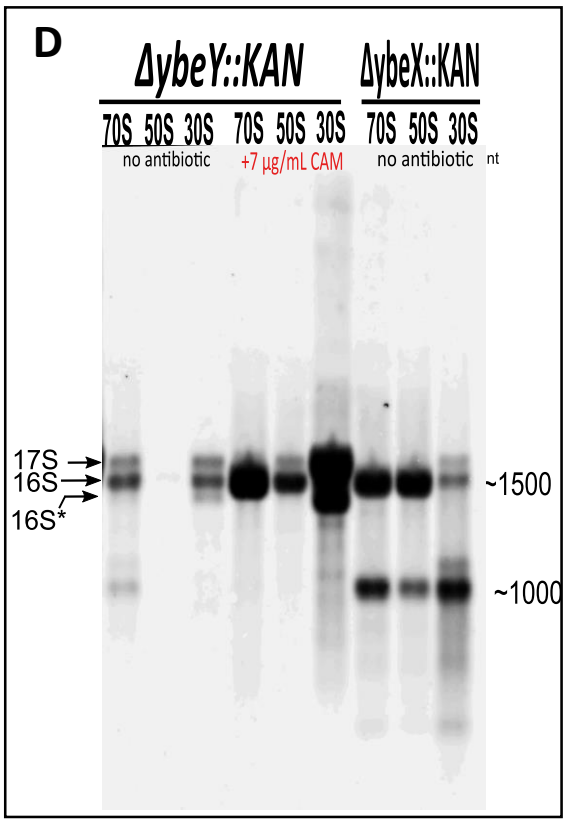
3.11 Effect of chloramphenicol on *ΔybeX* cells changes at late stages of growth

In the original protocol the cells are treated with CAM (7 µg/mL) when cell density at OD_{600} 0.2 (Siibak et al., 2009, 2011). This CAM concentration was set so OD_{600} fell 70% under chloramphenicol inhibition in comparison to untreated culture after 8h of treatment (Siibak et al., 2009). We grew wild type and *ΔybeX* cells under the same conditions, except that the start of chloramphenicol treatment was shifted to OD_{600} 0.7 (indicated as OD_{600} 0.7±0.1 on the figure). In the not treated controls the WT and ybeX cells exhibit similar ribosomal profiles where most material is in the 70S fractions (Figure 14B, upper row). In contrast, while CAM-treatment leads to some reduction of 70S ribosomes fraction in WT cells, it causes a much bigger reduction of 70S in the ybeX cells (Figure 14B , lower row).

3.12 Deletion of ybeX cause cells to accumulate distinct ribosome particles

The ribosome fractions purified from *ybeY* deletion strain showed distinct aberrant ribosomal RNA species from 17S rRNA precursors to misprocessed 16S rRNA, along with normal ribosomes containing mature 16S rRNA (Jacob et al., 2013). We did a Northern blot analysis of the RNA purified from 70S, 50S, and 30S ribosome fractions from three strains – *ΔybeX*, *ΔybeY*, and wild type. The comparison was made between chloramphenicol-treated and not treated bacteria.


A
WT
70S 50S 30S 70S 50S 30S
+7 µg/mL CAM
17S
16S
~1500
B
ΔybeX::KAN
70S 50S 30S 70S 50S 30S
No treatment
+7 µg/mL CAM
nt
17S
16S
~1500
~1000
C
ΔybeY::KAN
70S 50S 30S WT rRNA
nt
17S
16S
16S*
~1500
~1000


D
ΔybeY::KAN
ΔybeX::KAN
70S 50S 30S 70S 50S 30S
70S 50S 30S
no antibiotic
+7 µg/mL CAM
no antibiotic
nt
17S
16S
16S*
~1500
~1000

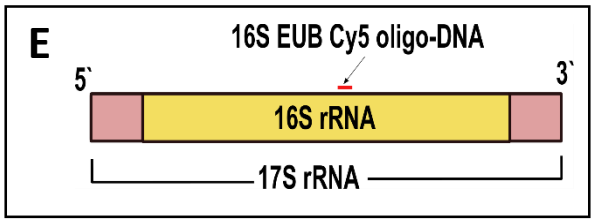

E
16S EUB Cy5 oligo-DNA
5`
3`
16S rRNA
17S rRNA

Figure 16 | Northern blot analysis of purified ribosomal RNA (rRNA) from sucrose gradient fractions. 3 µg (panel A, B, and C) or 5µg (panel D) of rRNA was used from purified 70S, 50S, and 30S rRNA as described in Material and Methods. Three E. coli strains – wild type (WT), *ΔybeX*, and *ΔybeY* - were grown in LB liquid media and bacteria were treated with 7 µg/mL chloramphenicol (Cam), indicated with red. The control growth contained no antibiotic treathment. (A) The rRNA originate from wild type (WT) E. coli strain, and (B) Ribosomal RNA from *ΔybeX* strain sucrose gradient fractions CAM treated and not treated cell.. (C) ribosomal rRNA originate from sucrose gradient fractions of *ΔybeY* strain loaded with wild type *E. coli* rRNA as a control purified from cells. (D) (E) The oligo used for hybridization was 16S rRNA specific Cy5 fluorescent dye labelled 16S EUB DNA-oligonucleotide. The scanning of the marked region on the membrane was done under different contrast set to make the visualisation better for the accumulated distinct rRNA species (shown on the edge of the gel with arrow ▷) in the lower panel partial membrane images in the boxes (with black arrows). The kanamycin resistance gene presence in *ΔybeY*, and *ΔybeX* strains shown as *::KAN*.

Intriguingly, while the gradient profiles of ybeX are similar to WT, while ybeY differs – the small subunit rRNA profiles are quite similar between ybeY and WT, while ybeX differs (Fig B). In late-growth ybeX cultures we see a major 16S rRNA cleavage species, which is ca 1000 nt long, and is present in the 70S fraction, which is traditionally considered consisting of translationally active ribosomes (or at least ribosomes active in translational initiation). As the 1000 nt fragment originates from the 3' – side of the 16S rRNA, which contains the Shine-Dalgarno region needed for efficient translational initiation, this result certainly presents an enigma. The 1000-nt fragment is present regardless of the CAM-treatment and can also be found in lysates prepared instantly by hot-phenol treatment – making it unlikely that it originates in some post cell-harvesting artefactual RNA cleavage (data not shown).

4. DISCUSSION

When a microbiologist steps in a lab, among the first things would be streaking cells on food containing plates. In our case this is LB-Agar media poured on the petri-dishes. Colony formation of a strain can represent the biology behind the genetic composition of the strain, like how the case is for *ybeX*, *ybeY* and *ybeZ* deletion mutant strains in comparison with WT *E. coli* strain (Figure 7). Deletion of *ybeY* in E. coli causes a dramatic effect on growth of colonies. The deletion of ybeX and its effect on the colony formation is a complex growth phenotype, which comes out only under certain conditions (Figure 7). This bring out a complicated phenotypic scenario which was studied further.

A heterogenic growth might be caused by the depletion of *ybeX* or just growth media dependent. This consistent cellular heterogeneity in growing bacterial culture came out in the growth assays clearer. The longer lag phase is always present after overnight culture diluted into fresh LB media for both ΔybeY and ΔybeX strains (Figure 8). The growth rate of the *ybeX* deletion mutant in comparison to WT behave similarly to wild type rather than *ΔybeY*. the cells are growing exponentially as in wild type when they are diluted from exponential phase (Figure 8A). Here we understand that the phenotype most notable occur when the cell growth slow down and cells are prepared to be entered to stationary phase.

Biogenesis of a ribosome is expensive for growing cells as synthesis of ribosomal components compromises a large portion of protein translation and most of the transcriptional activity. But it was shown that more than half of the ribosomes which are made during the exponential growth are degraded in *E. coli* when cell growth is slowed down prior to entry to stationary phase (Piir et al., 2011). This is where both deletion strains give the strongest phenotypes.

Electrophoretic analyses of purified defective 70S ribosomes from both the *ΔybeY* and kasugamycin-treated *E. coli* abnormal ribosomes are consist of 17S rRNA precursor or misprocessed 16S rRNAs along with normal ribosomes containing mature 16S rRNA (Jacob et al., 2013). In our lab we got essentially very similar results. The 70S fractions originating from *ybeY* deletion strain accumulate a distinct rRNA species that is approximately 1000 nt long. The 17S rRNA is present in both *ΔybeY* and CAM-treated *E. coli* ribosome fraction (Figure 16C). Ribosomes are known to be mostly stable macromolecules and fully processed, although their degradation in growing bacteria has been shown (Maiväli et al., 2013; Piir et al., 2011).

The chloramphenicol treatment quantitatively decreased the amount of 17S rRNA in *ΔybeY* treated cells ribosomes (Supp. Fig. S11) and distinct rRNA species did not appear (Figure 16). The cell lysate preparation for sucrose gradients were done under the same conditions. Regarding that occurrence of the distinct 16 rRNA species in growing *ΔybeY* 70S ribosomes seems to be growth dependent rather than an experimental artefact. Chloramphenicol freeze the ribosomes meaning that the growth has slowed down (Lazzarini and Santangelo, 1968). Because of that *ΔybeY* would never reach the growth phase where the distinct rRNA species are appearing.

Indeed, overexpression of YbeY seems to have a compensatory effect on the *ΔybeX* strain phenotype. Recent studies give an evidence about YbeY in the processing of the 3`- end of 16S rRNA together with ribosome factor Era (Ghosal et al., 2018). Era is one of the interaction partners of YbeY (Vercruysse et al., 2016) (Supp. Fig. S4), it was experimentally (B2H system) shown. Overexpression of one ribosomal factor (RbfA, YjeQ (Supp. Fig. S11-13), Era, RimM) could potentially compensate for loss of another factor (Thurlow et al., 2016). Recently it was shown that overexpression of ribosome-associated GTPase Era in *ΔybeY* improved the growth, 16S ribosomal RNA processing and 70S ribosome assembly in *Escherichia coli* (Ghosal et al., 2018). We discovered that overexpression of YbeY in *ΔybeX* lead to the similar effects. This could mean that both genes are plays role together in the processing or maturation of the ribosome that overexpression of one could partially rescue the phenotype of other. Now, we are studying further the overexpression of ribosomal factors in both deletion strains via overexpression plasmids from *E. coli* ORFeome plasmid library (Rajagopala et al., 2010).

Accumulated rRNA species in overnight grown *ΔybeX* cells are disappeared when the cells are diluted into fresh media (Figure 12 and Figure 14). The longer lag phase which was observed for *ΔybeX* "Stationary" cells inoculated into fresh media (Figure 8B) might cause of accumulated 17S rRNA and degradation products. The lag phase is known to be a preparatory stage of exponential growth. And indeed this is the case for the longer lag phase in *ΔybeX*. The accumulation occurs again when cells enter the stationary phase.

As a control experiment for the accumulated rRNA in ribosome fractions and CAM-treatment (*ΔybeY*) we tested two ribosomal factor (RbfA and YjeQ) deletion mutant's strains. The CAM treated cells did not accumulated CAM-particles (data not shown, similar to Sup. Fig. S10A) . The 70S fractions of *ΔyjeQ* clearly have only 16S mature ribosomal RNA in both treated and not treated bacteria (Figure S12-S13).

Functional Interactions of the Metalloprotein YbeY, Involved in Ribosomal Metabolism, with The Putative Metal Efflux Protein YbeX

Ismail Sarigül

SUMMARY

Ribosomes are macromolecular complexes that are responsible of protein synthesis in all cells. In bacteria ribosomes are made of 3 ribosomal RNAs (16S, 23S, 5S) and 51 ribosomal proteins that fold on the RNA to accomplish the ribosmal subunits; small ribosomal subunit (30S) and large ribosomal subunit (50S). An actively translating 70S ribosome is made of assembled 50S and 30S ribosomal subunits.

YbeY is a putative ribosomal endoribonuclease which has been implicated, among other things, to be involved in quality control of 70S ribosomes, in 17S pre-rRNA maturation and in ribosomal degradation. However, controversy reigns over its mode of action, substrates, co-factors, and interaction partners.

In many bacteria *ybeY* is located in the *ybeZYX-Int* operon, where *ybeZ* encodes a PhoH subfamily protein with NTP hydrolase domain and *ybeX* encodes a putative Cobalt/Magnesium efflux protein. We aimed to study the deletion mutant strains of *ybeZYX-Int operon* to gain insight on YbeY`s conserved role within the conserved operon.

Deletion of *ybeY* causes a growth defect in *E. coli*, so the colonies formed on LB Agar plates are smaller than wild type. In contrast, deletion of *ybeX* leads to a variety of colony sizes, when the cells are plated directly from a glycerol stock. While deletion of *ybeY* has a reduced growth rate during normal exponential phase growth, ***Δ****ybeX* and ***Δ****ybeZ* exhibit no effect on exponential growth. Interestingly, Δ*ybeX* stationary phase inoculum, but not exponential phase inoculum cells, exhibit a prolonged lag phase in relation to WT and *Δybe*Z cells.

Nevertheless, ***Δ****ybeY* and ***Δ****ybeX* have largely overlapping phenotypes, including accumulation of 17S pre-rRNA and an approximately 1 kb 16S rRNA cleavage product (cleaved from the 3' side), sensitivity to heat shock, and to the protein synthesis inhibitors chloramphenicol and erythromycin. Overexpression of the YbeY partially rescues the phenotype of ***Δ****ybeX*.

Taken together our results indicate that both mutant strains, ***Δ****ybeX* and ***Δ****ybeY,* are defective in rRNA maturation, and this could be a sign to a functional interaction between *ybeY* and *ybeX*.

Key words: YbeY, YbeX, E. coli CorC, Ribosome, 16S rRNA, bacteria

Ribosomaalses metaboolis osaleva metalloproteiini YbeY funktsionaalne koostoime koos võimaliku metalli efluxproteiiniga YbeX

Ismail Sarigül

KOKKUVÕTE

Ribosoomid on makromolekulaarsed kompleksid, mis vastutavad valkude sünteesi eest kõigis rakkudes. Bakterite ribosoomid koosnevad 3 ribosomaalsest RNA-st (16S, 23S, 5S) ja 51 ribosomaalsest valgust. Ribosoom koosneb kahest ebavõrdsest alamühikust: väike alamühik (30S) ja suur alamühik (50S). Aktiivne 70S ribosoom moodustub 50S ja 30S alamühiku ühinemise käigus.

YbeY on oletatav ribosomaalne endoribonukleaas, mis on muu hulgas seotud 70S ribosoomide kvaliteedikontrolliga, 17S rRNA küpsemise ja ribosoomide lagundamisega, kuigi siin on palju vaidlusi töömehhanismi, substraatide, kaasfaktorite ja nende koosmõju kohta.

Paljudes bakterites paikneb *ybeY ybeZYX-Int* operonis, kus *ybeZ* geen kodeerib NTP hüdrolaasi domeeniga PhoH alamperekonna valku ja *ybeX* geen kodeerib oletatava koobalti / magneesiumi väljavoolu valku. Meie eesmärgiks on uurida *ybeZYX-Int* operoni deletsioonimutantide tüvesid, et saada paremat aimu YbeY-i konserveerunud rollist. *ybeY*-i eemaldamine põhjustab *E. coli* kasvufaasi defekte, nii et LB agari plaatidel tekkivad kolooniad on väiksemad kui metsikut tüüpi rakkude puhul. Vastupidiselt sellele põhjustab *ybeX-i* deletsioon kolooniate suuruste variatsioone, juhul kui rakud plaaditakse otse glütseroolivarust. Kuigi *ybeY* deletsioonil on normaalse eksponentsiaalse faasi kasvu ajal vähenenud kasvukiirus, ei mõjuta *ΔybeX* ja *ΔybeZ* eksponentsiaalset kasvu. Huvitav on see, et Δ*ybeX* statsionaarse faasi inokulaadil, kuid mitte eksponentsiaalse faasi inokulaadirakkudel, on pikaajalisem lag faas võrreldes WT ja *Δybe*Z rakkudega.

Sellegipoolest on *ΔybeY*-l ja *ΔybeX*-l on suurel määral kattuvad fenotüübid, kaasa arvatud 17Si pre-rRNA ja umbes 1 kb 16S rRNA lõhustamisprodukti (lõhustatud 3'-küljest) kogunemine, tundlikkus kuumašoki ja valgusünteesi inhibiitorite kloramfenikooli ja erütromütsiini suhtes. YbeY-i üleekspressioon suudab osaliselt kompenseerida *ΔybeX* kasvufenotüüpi. Kokkuvõttes meie tulemused näitavad, et *ΔybeY* ja *ΔybeX* tüved on defektsed rRNA küpsemise suhtes, ja see võib olla oluline samm, et aru saada funktsionaalsest interaktsioonist *ybeY* ja *ybeX* vahel.

Võtmesõnad: YbeY, YbeX, E. coli CorC, Ribosoom, 16S rRNA, bakterid

CONCLUSIONS

1. Depletion of *ybeY* and *ybeX* lead to profound overlapping phenotypes during *E. coli* late exponential and early stationary growth.
2. Ribosome biogenesis is impaired in both Δ*ybeY* and Δ*ybeX E*. coli strains.
3. *Δ*ybeX* phenotype is highly complex and depends on growth conditions.
4. Our results suggest the possibility that YbeY and YbeX work in the same pathway.

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WEBPAGES AND SOFTWARE

Protein Data Base (PDB), YbeY crystal structure:

https://www.rcsb.org/structure/1xm5 (28/06/2019)

SnapGene® software (GSL Biotech, available https://www.snapgene.com/

PDB,

PDB, The cystathionine-beta-synthase (CBS) domain of magnesium and cobalt efflux protein CorC in complex with both C2'- and C3'-endo AMP https://www.rcsb.org/structure/5YZ2

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DataAcquisition Program (Indrek Must, University of Tartu Institute of Technology core facility for apparatus engineering)

ANNEX 1

SOLUTIONS

10x MOPS buffer

200 mM morpholineopropanesulfonic acid (MOPS)

Adjust pH to 7.2 with 2N NaOH

20mM sodium acetate (20 mL of stock solution 1M NaAc pH 7.0)

10 mM EDTA (20 mL of stock 0.5M pH 8.0)

Filter through 0.2 µm filter and protect from direct light. Store at room temperature.

20x SSC

3M NaCl

0.3M sodium citrate

Adjust pH to 7.0 with HCl (at 20°C)

Filter through 0.2 µm filter and autoclave.

Store at room temperature.

10% SDS

Add 50 g (w/v) of SDS to warmed up (65°C) water 400 mL mQ water.

Dissolve

0.5M EDTA pH 8.0

Add 93.06 g EDTA to 400 mL of MQ. Add NaOH pellets while stirring to bring the pH to 8.0 (EDTA will completely dissolve when pH will be around 8). Filter through 0.2 µm filter and autoclave.

Stripping Solution (500 mL)

490 mL mQ water

5 mL EDTA (0.5M pH 8)

5 mL 10% SDS

10xTEN

10 mM EDTA

100 mM Tris-HCl (pH 7.0)

1M NaCl

0.5M Sodium Phosphate Buffer (100 mL pH 7.2)

Approximately 70 mL of 0.5M Na_2HPO_4 prepared,

The pH 7.2 was set with ~30 mL of 0.5M NaH_2PO_4

Filter through 0.2 µm filter and autoclave. Stored at 4-8°C.

Hybridization Buffer

0.5 M Sodium phosphate buffer pH 7.2 containing 7% SDS (14g SDS dissolved in 200 mL of 0.5 M $NaHPO_4$ buffer. Heat in water bath at 65°C occasionally swirling the bottle to dissolve the SDS)

Wash Buffer

20 mM Sodium phosphate buffer containing 1% SDS

100 mL of 10% SDS solution and 40 mL of 0.5 M Sodium phosphate buffer (pH 7.2) were measured and mixed in cylinder, final volume was taken up to 1L with MQ water.

Name of the Oligo	Place/location	Sequence
17S_5prim_Cy5	17S rRNA 5`end / 3L-33L	5`-Cy5-AGT TTG ACG CTC AAA GAA TTA AAC TTC GTA-3`
17S_3prim_Cy5	17S rRNA 3`-end / 1T-30T	5`-Cy5-GTG AGC ACT ACA AAG TAC GCT TCT TTA AGG-3`
16S_EUB_338_Cy5	16S rRNA /inside 338-356	5`-Cy5-GCT GCC TCC CGT AGG AGT-3`
16S_3prim_Cy5	16S rRNA /3`end 1529-1543	5`-Cy5-TA AGG AGG TGA TCC -3`
23S_190_Cy5	23S rRNA /inside 163-185	5`-Cy5-GGT TCG CCT CAT TAA CCT ATG G-3`
23S_h25_Cy5	23S rRNA /inside 527-552	5`-Cy5-CGC CTA AGC GTG CTC CCA CTG CTT G-3`

Table A1 |The sequences of used Cy5 labelled DNA oligonucleotides their name and locations on the rRNA, T- terminus and L- leader to the rRNA.

ANNEX 2

Northern Hybridization (RNA Blot)

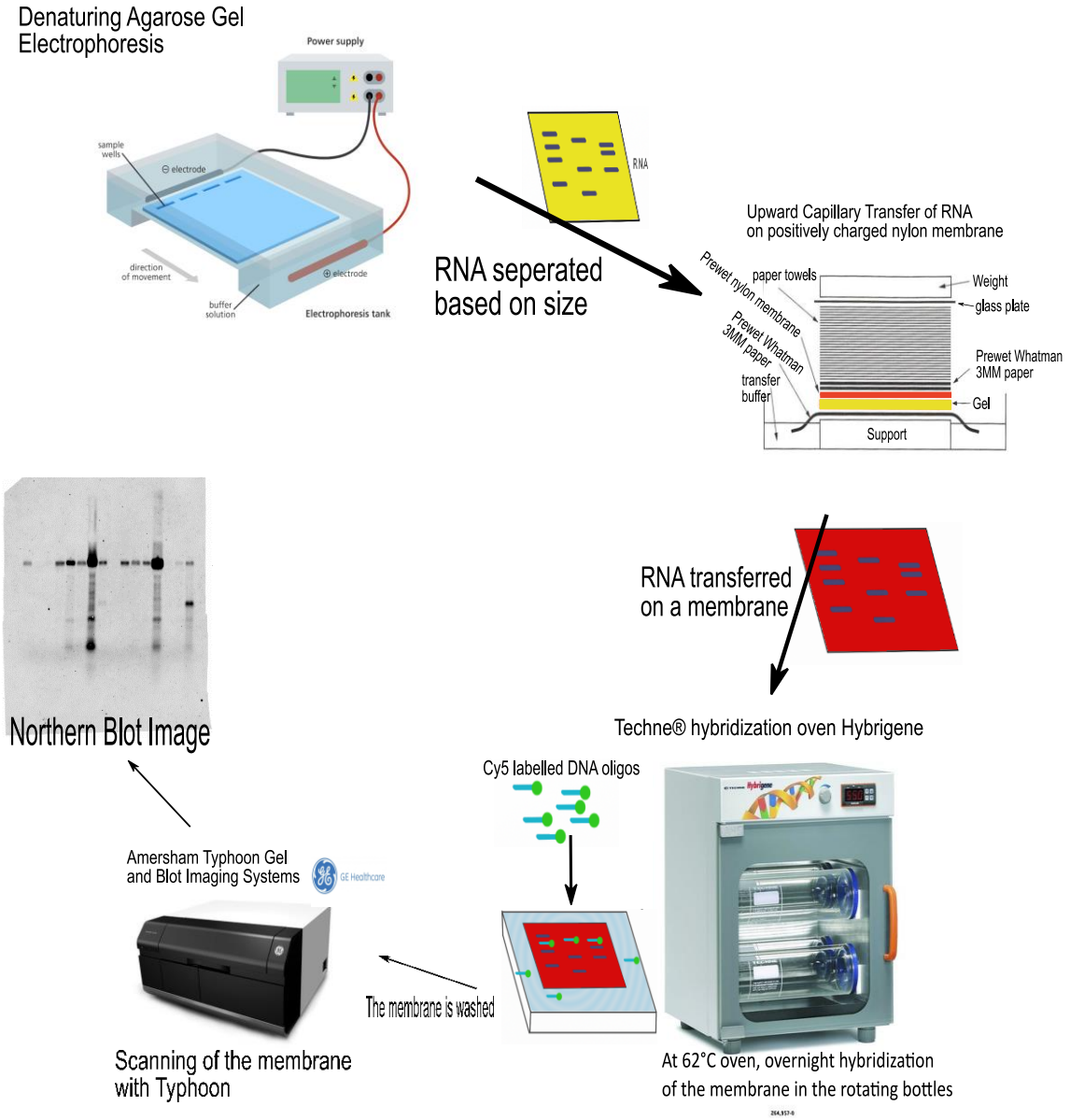

Figure A1 | Schematic depiction of the Northern blot analysis in general. Denaturing agarose gel electrophoresis is done to separate the RNA species based on their sizes. Then, the agarose gel (shown in yellow) replaced on a capillary transfer platform and the transfer of RNA to the charged nylon membrane was done overnight. The membrane was stored between two Whatman paper pre-wetted in 10x SSC buffer or proceed on the membrane hybridization with specific oligonucleotides. The membrane was replaced in a temperature controlled incubated in a rotating bottle at 62°C.

ANNEX 3

Colony PCR (Polymerase Chain Reaction)

Reagents	**Amount**
5x HOT FIREPol Blend Master Mix	5 µl
$MgCl_2$ (0,5 mM)	0,25 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
Cell suspension (in MQ)	5 µl
MQ water	Upto 25 µl

PCR was made by using 5x HOT FIREPol Blend Master Mix, which is containing all required components including $MgCl_2$ for PCR, except water, primers, and template (DNA). A colony from each plate (WT and ΔybeX LB- plates) was picked and dissolved in 50 µl of MQ water. The following was added to each PCR tube for PCR: 13 µl of MQ with 5 µl of 5x HOT FIREPol Blend Master Mix, 1 µl of each primer (in total, 2 µl of primers), 5 µl of dissolved cells.

EPPENDORF mastercycler gradient thermal cycler 96 well PCR machine was used as a thermocycler to create the necessary conditions for PCR. The installed settings:

PCR settings			
Initial Denaturation	**95°C**	**5 min**	
Denaturation	95°C	1 min	**30 cycles**
Primer Annealing	56 °C	1 min	
Extension	72 °C	1 min	
Final Extension	**72 °C**	**10 min**	

5 µl of each samples were loaded directly on 1,2% agarose gel. GeneRuler 1 kb plus was used as a marker.

ANNEX 4

SUPPLEMANTARY INFORMATION

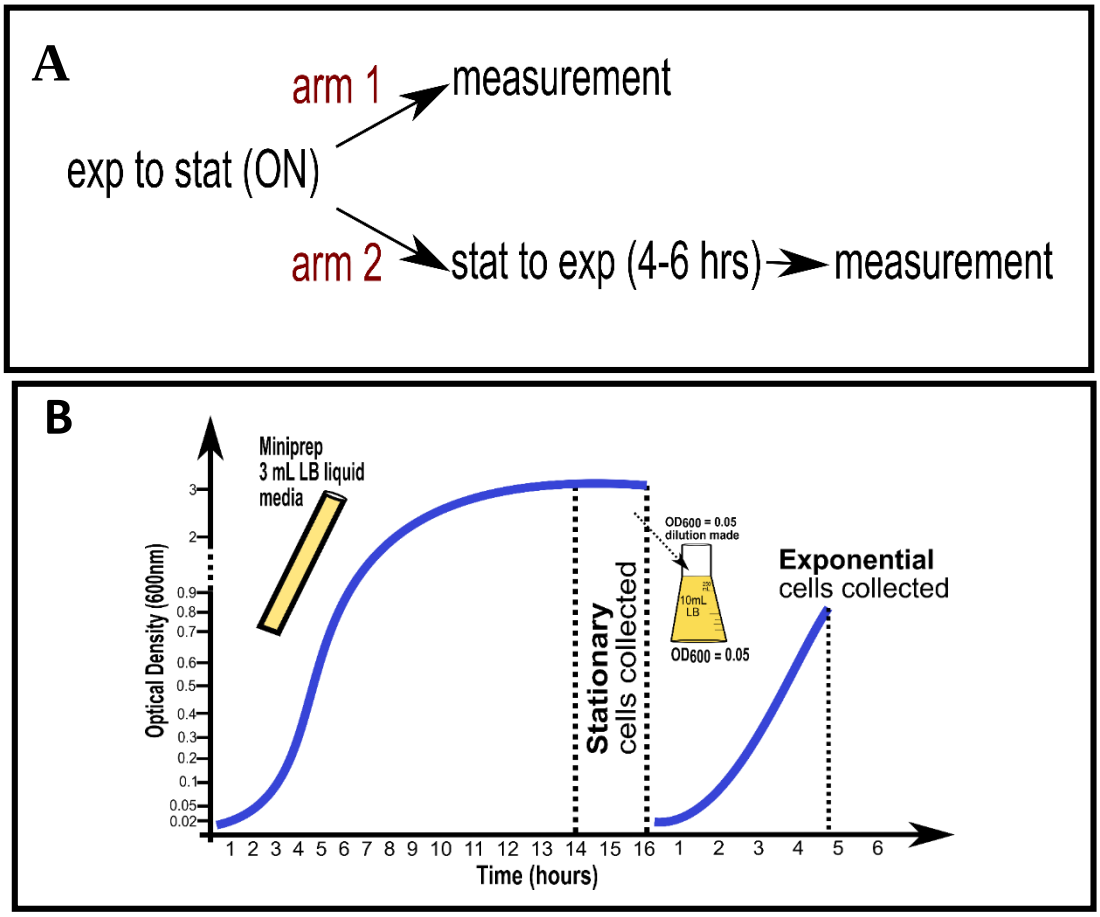

Figure S1 | (A) A scheme for the experimental set up for 96-well plate growth and dot spot experiments. (A)In the first arm of the experiment the cells were grown over-night (ON) in 3 ml minipreps from exponential phase (exp) to stationary phase (stat). The growth measurement either involves diluting cultures into fresh LB in 96-well plates (shown in Figure 8 panel B, C), or making serial dilutions onto LB-agar plates (Figures 9-10). In the second arm the stationary cells were, before measurement, diluted into liquid LB medium, grown there back into exponential phase. Arrows denote dilutions of cultures. (B) The representative visual scheme is shown. Cells were grown in LB liquid media for 14-16 hours. Following morning the bacteria cultures were diluted in fresh media (OD_{600} 0.05) in a flask grown bavk to exponential phase. Blue curve represents the growth of bacteria in LB liquid media over the time. x- axis show the time in hours and y- axis show the monitored cells density OD600.

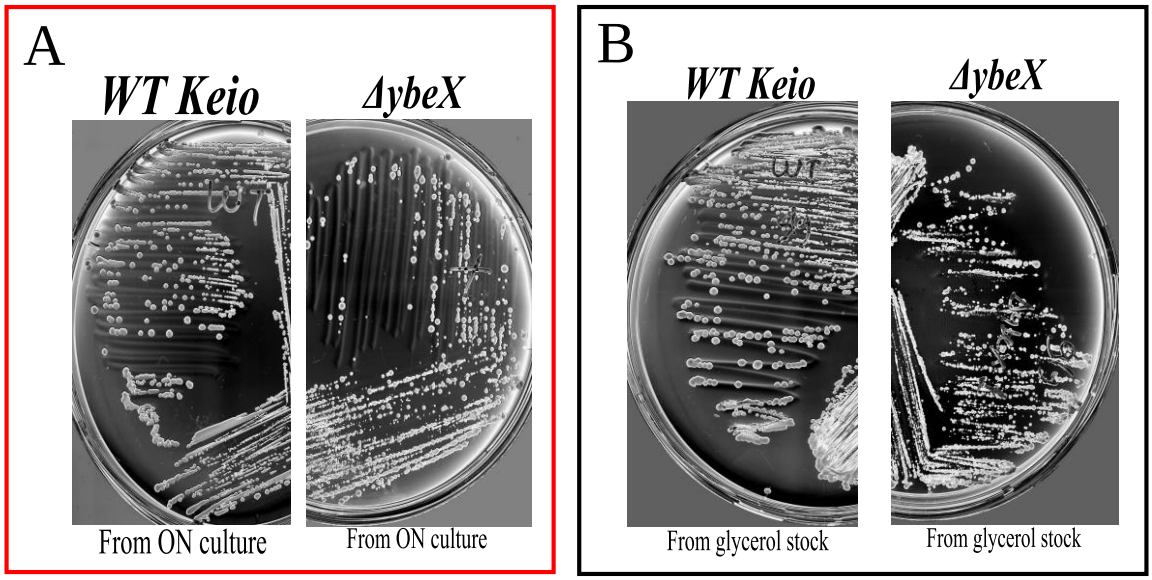

Figure S2 | Colony growth of the *wild type E. coli* and *ybeX* deletion mutant. (A) A colony from the WT and *ΔybeX* plates were transferred into 3 mL LB liquid media and grown overnight in the shaking (220 rpm) incubator at 37°C. Following morning the bacteria were streaked directly on the LB-Agar plates, and the plates incubated at 37°C incubator. (B) Glycerol stocks from these overnight cultures were prepared, and the cells were streaked on the LB-Agar plates from glycerol stocks. LB-Agar plates were incubated at 37°C incubator for 14-16 hours.

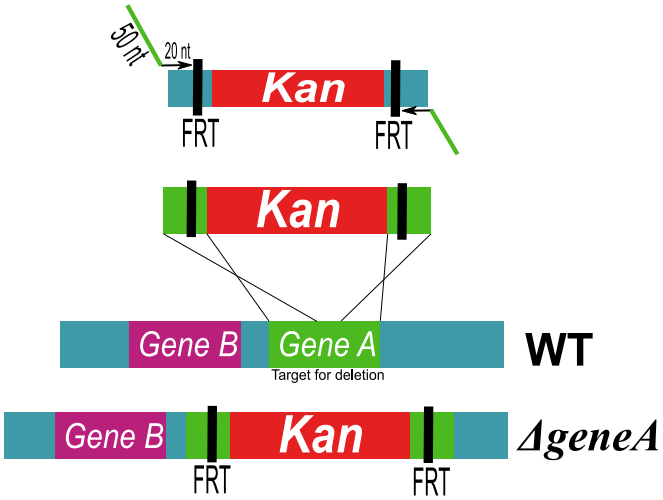

Figure S3 | Construction of Keio collection, single-gene deletion mutant strain library (Baba et al., 2006; Datsenko and Wanner, 2000)**.** PCR primers are designed to make the gene knockout with homologous recombination-based technique which requires the phage lambda (λ) Red recombinase for disturbing E. coli chromosomal genes. Gene knockout primers are designed so 20-nt (3` ends) for priming upstream and downstream of the FRT sites flanking the kanamycin resistance gene in pKD13 plasmid (black arrows located near FRT regions) contain the resistance gene. The green lines represent 50-nt of 5` ends homologous to upstream and downstream to chromosomal sequences of interest target gene for deletion.

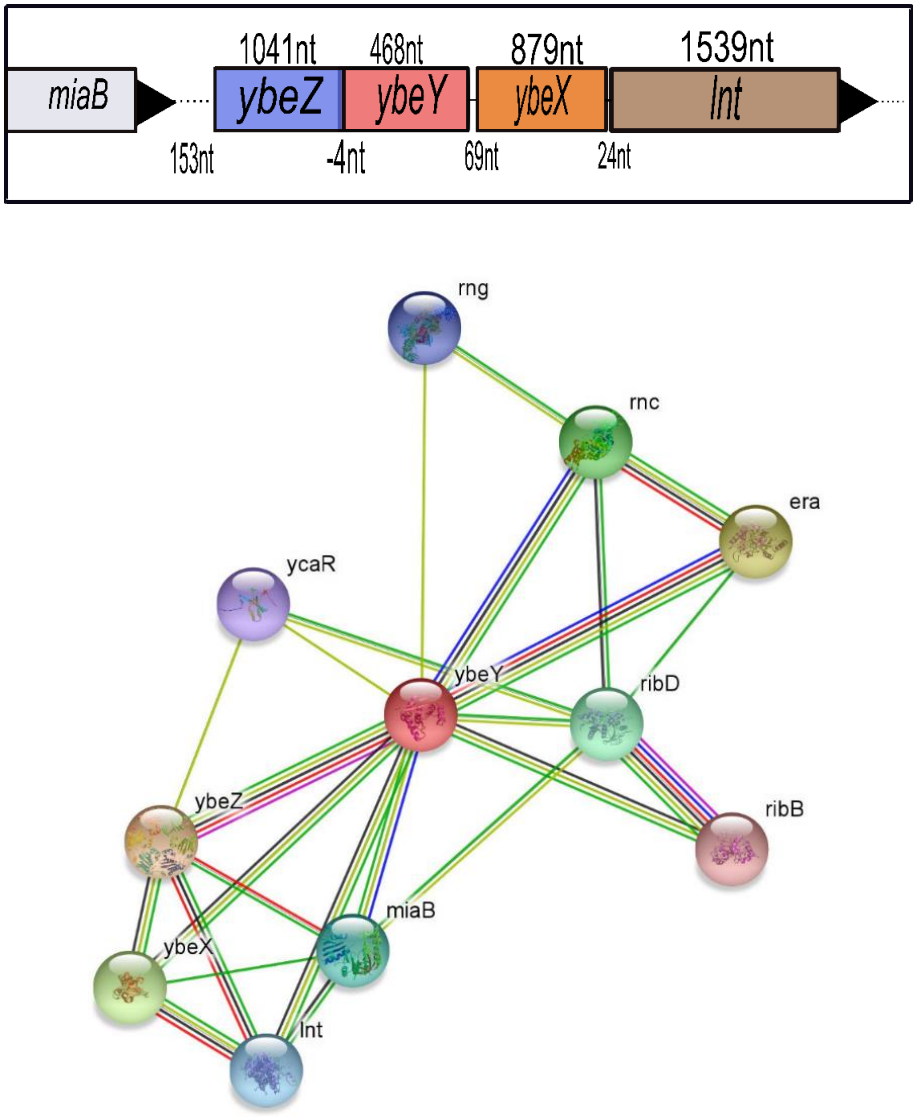

Figure S4 | (A) *ybeZYX-lnt* operon in *Escherichia coli* and . Genes; *ybeZ* (blue), *ybeY* (red), *ybeX* (orange), and *lnt* (brown) and their size is given above the genes sequently 1041,468,879, and1539 nucleotide(nt). End of ybeZ is overlaped 4 nucletide with start of ybeX annotaed below the genes as -4nt. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database predicted protein protein interactions are shown. Available 3D structures of the proteins are given in the nodes. Lines on the interaction scheme are represent; Green lines ~ gene neighborhood, red lines ~ gene phusion, blue ~ gene co-occurance, black ~co-expression, yellow (light green) ~ texmining. Purple lines show known interactions.

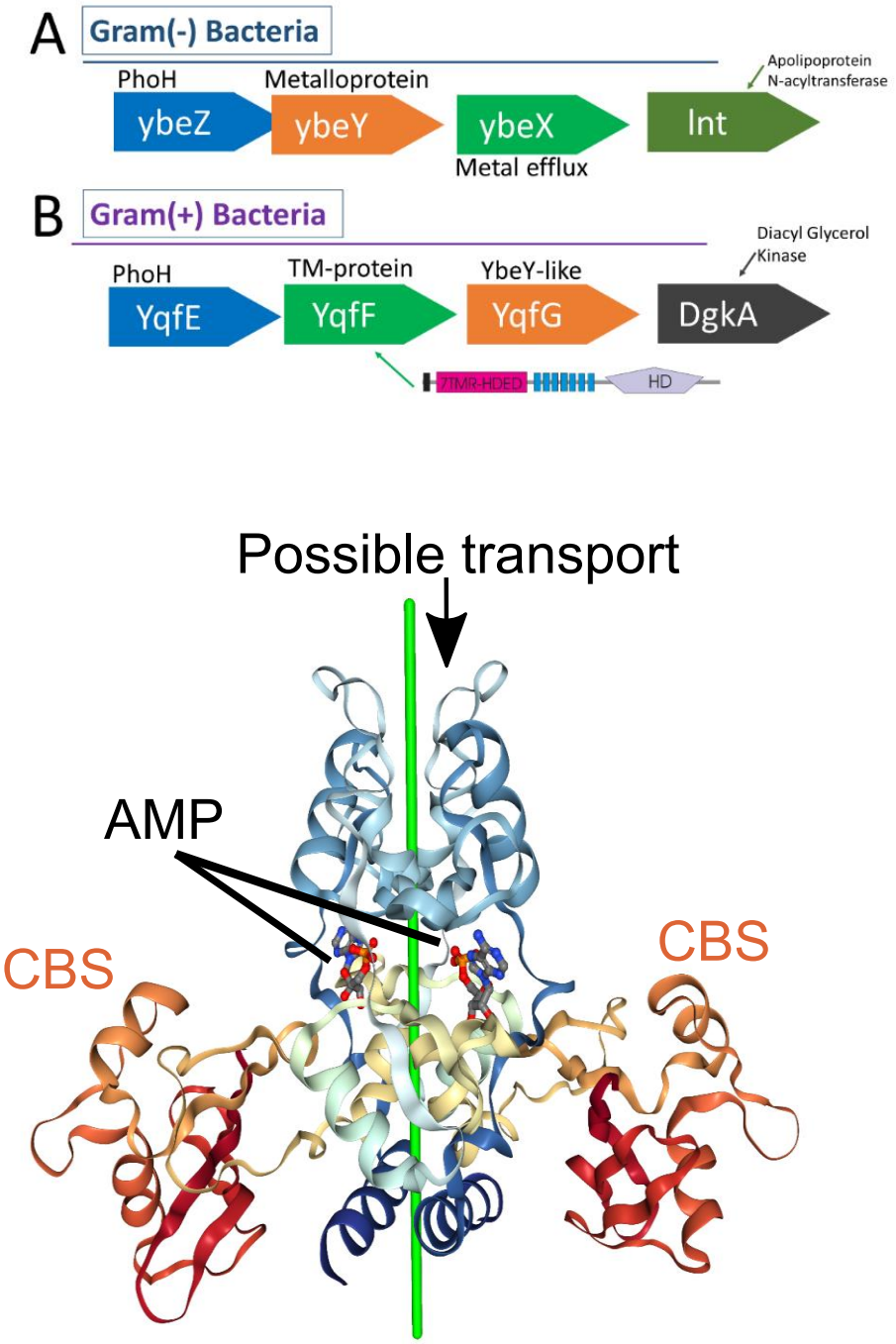

Figure S5 | Conserved ybeY gene operons ybeZYX-lnt (in Escherichia coli) and yqfEFG-dgkA (in Bacillus subtilis. Gene colors are represent the analogy of *ybeZ-yqfE*(blue), ybeX-yqfF(green), and the homology between ybeY and yqfG (orange) (Baumgardt et al., 2018). Other genes are shown lnt (brown). End of ybeZ sequence is overlap 4 nucletide with start of ybeY in E. coli and many other bacteria. The putative transporter ybeX (E. coli CorC protein) crystal structure is shown. A dimer form of ybeX with 2 CBS domain and AMP molecules which might be involved in regulation are indicated.

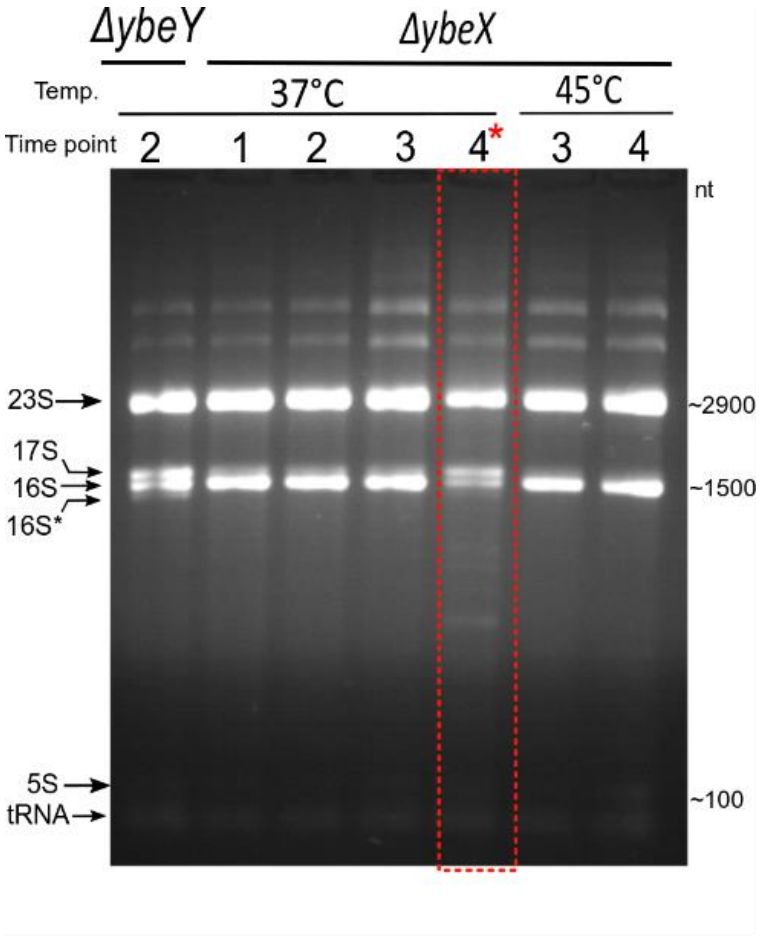

Figure S6 | Analysis of total RNA from *ΔybeY*, and *ΔybeX* strains on 1.8% agarose gel electrophoresis. The *ΔybeX* cells were grown in LB liquid media at 37°C or 45°C shaker and cell density was monitored via measuring OD_{600} units. Indicated time points are: 1 - OD_{600} = 0.2; 2 - OD_{600} = 0.3; 3 - cells were grown for additional 30 minutes at indicated temperatures (from the 2. Time point); 4 - cells were grown for a total of 1h (from the 2. Time point). The total RNA was purified with hot phenol extraction. The positions of 23S, 17S, 16S and 16S* rRNAs are indicated based on their mobility on the agarose gel. The red box indicates the only time point where heterogeneous degradation products together with accumulated 17S rRNA can be observed in *ΔybeX* cells. Purified *ΔybeY* total RNA was loaded as a mobility control for 16S, 17S and 16S* rRNAs.

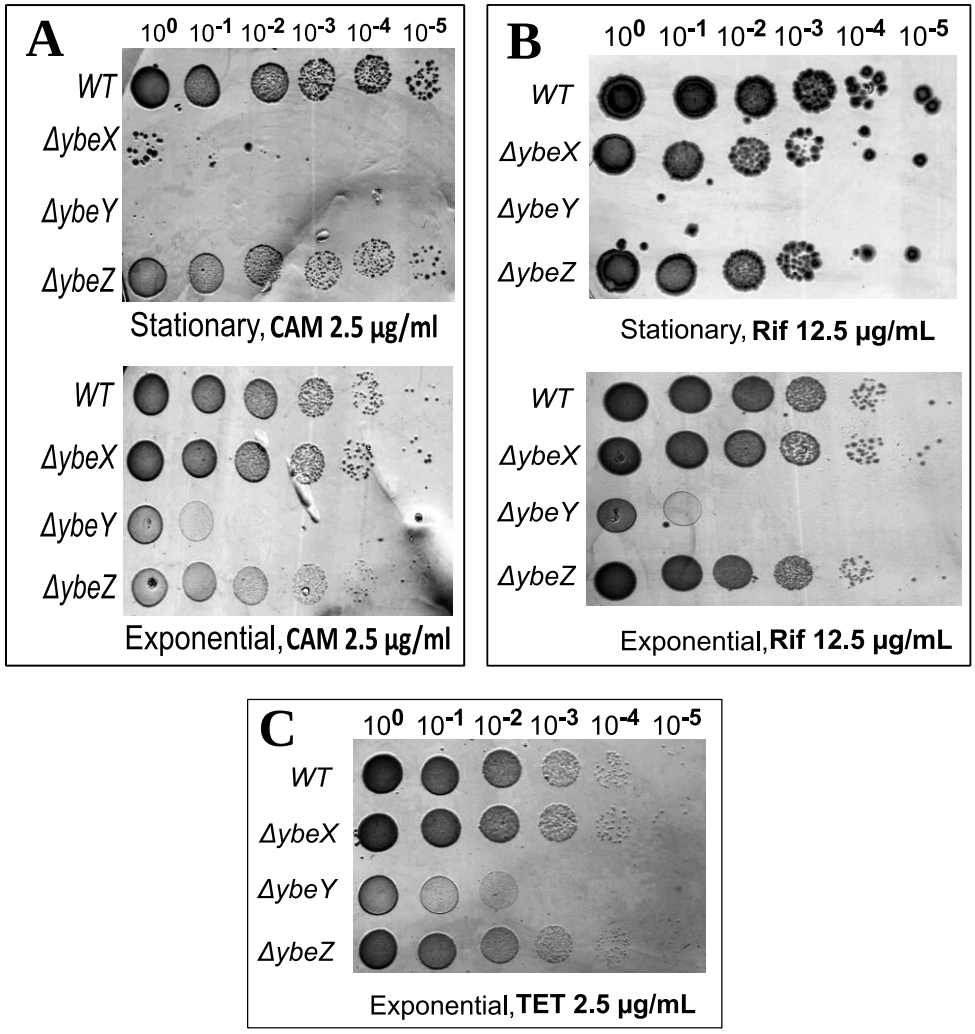

Figure S7 | Dot-spot experiment of antibiotic sensitivity. Wild type (WT) *E. coli* and *ybeZYX-lnt* operon gene deletion mutants at (A) 2.5 µg/mL chloramphenicol (CAM) (B) 12.5 µg/mL rifampicin (Rif) (C) 2.5 µg/mL tetracycline (TET) antibiotics containig plates are shown from 2 different growth phase, Exponential and Stationary. The data for the cells were collected as it is explainded in Figure 8A. Shortly, Wild type and deletion mutant strains were grown in LB liquid media overnight and cells were used for Stationary plate experiments. Re-grown cells to exponential phase were used for Exponential plate experiemnts.

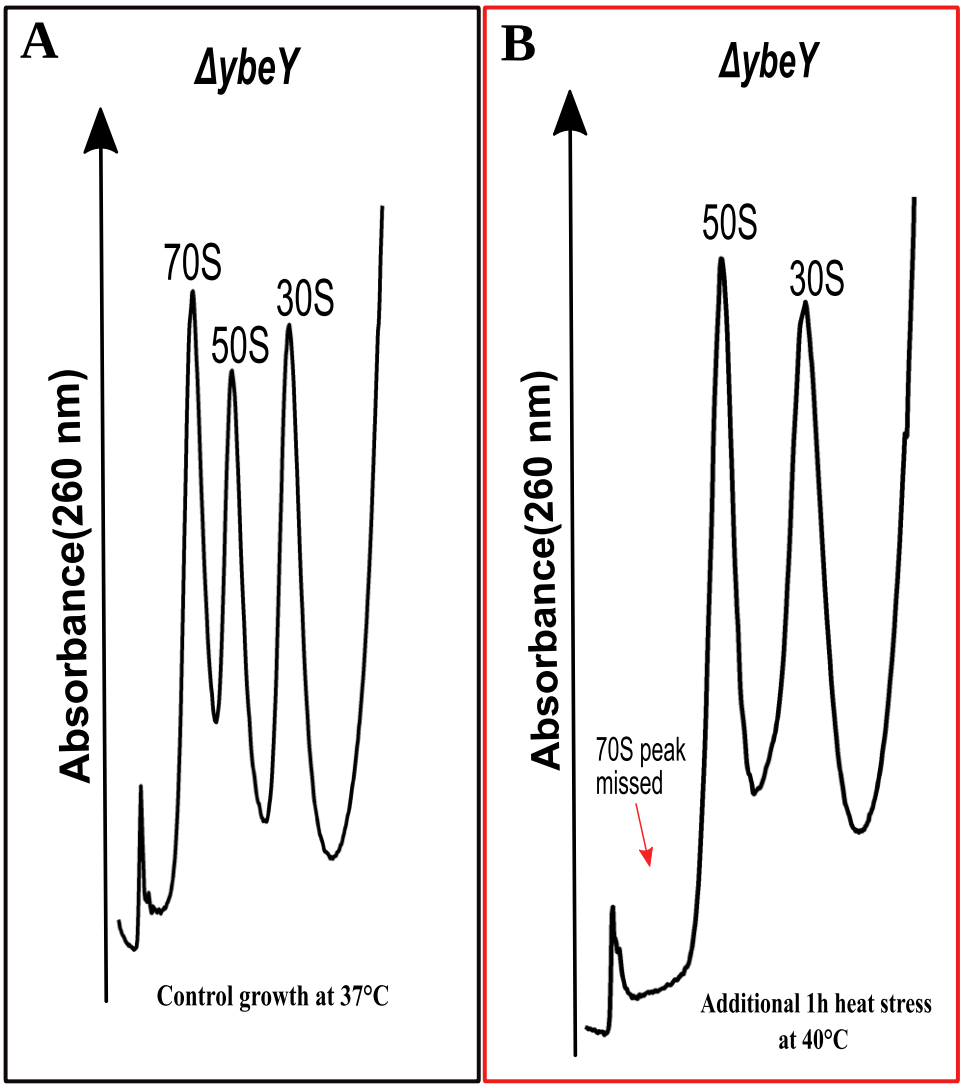

Figure S8 | ΔybeY cells have no thermotolerance. (A)10-40% sucrose gradient sedimentation profiles of ΔybeY at 37°C (OD_{600} 0.3 + 1h) as acontrol and (B) 1h treatment at 40°C after cell density has reached 0.3. The cells were grown in LB in presence of Kan (25 µg/mL) at 37°C till OD_{600} reached 0.3, and then the culture were split to two flask and one of the flaskes were incubated for 1 hour at 40°C. Bacteria were lysed and lysate clarified with centrifigution. 50U of lysate was loaded on the top of the prepared sucrose gradients. Proceed the process as it is explained in material and methods.

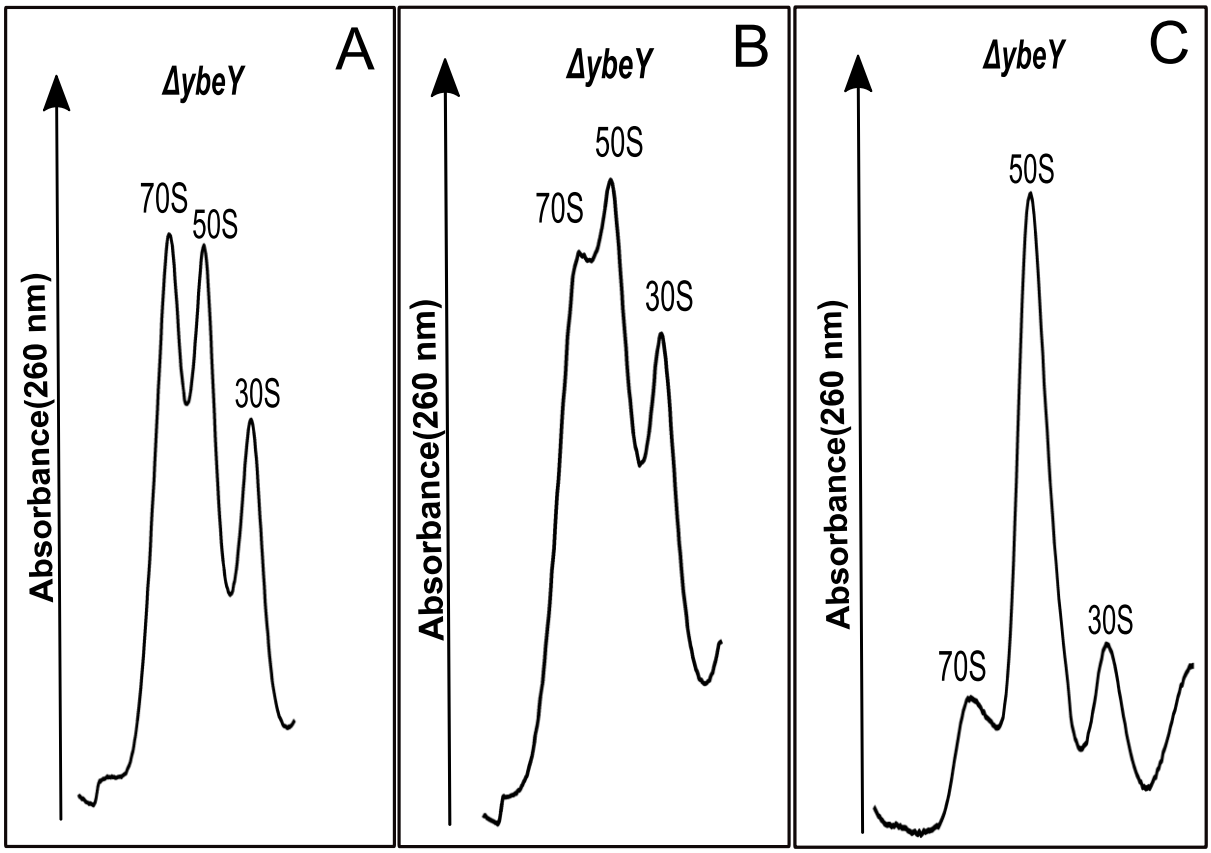

Figure S9 | Deletion of ybeY causes decrease in the active 70S ribosome fraction over the time. 10-30% sucrose gradient sedimentation profiles of ΔybeY grown in LB liquide media at 37°C. The cells were harvested at different stage of growth (conducted via measuring OD_{600}) (A) OD_{600} 0.2 (B) OD_{600} reached 0.6±0.1 (C) additional 2h of growth after OD_{600} reached 0.3.

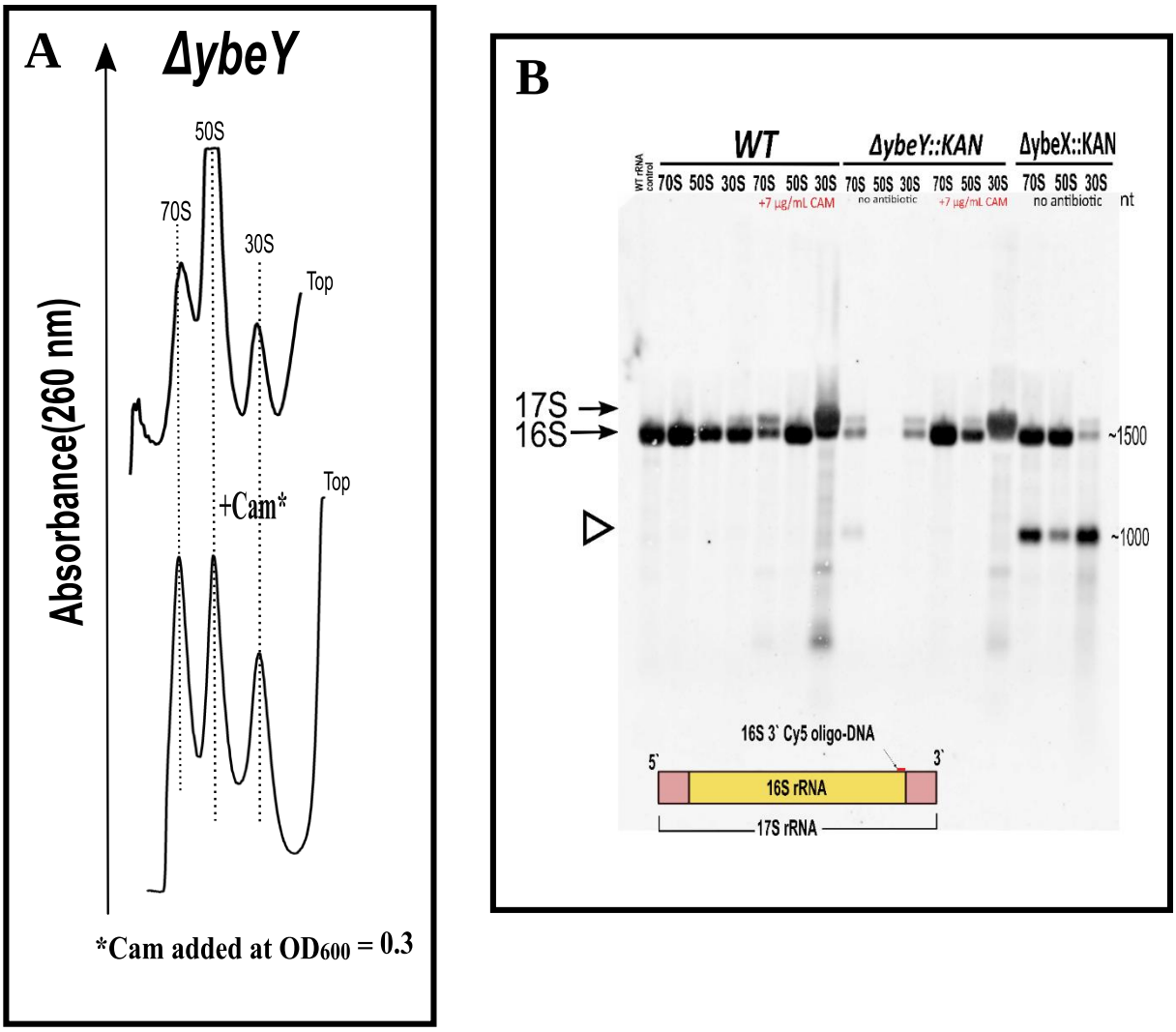

Figure S10 | *ΔybeY* cells do not accumulate chloramphenicol (Cam, 7 µg/mL) particules. (A)10-30% sucrose gradient sedimentation profiles of ΔybeY at 37°C (OD_{600} 0.3 + 2h) as acontrol and 2h of Cam treatment after cell density has reached 0.3. The cells were grown in LB at 37°C till OD_{600} reached 0.3, and then the culture were split to two flask and one of the flaskes were incubated in presence of Bacteria were lysed and lysate clarified with centrifigution. 50U of lysate was loaded on the top of the prepared sucrose gradients. Proceed the process as it is explained in material and methods.

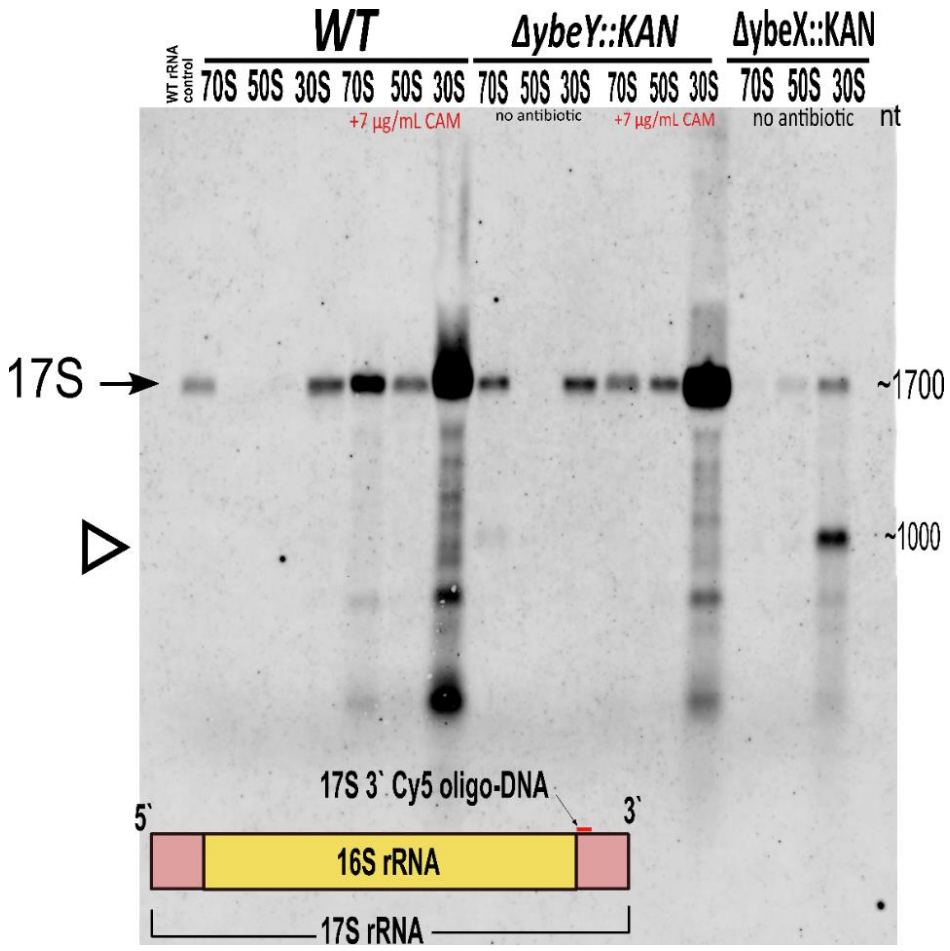

Figure S11 |Northern blot analysis of purified rRNA from sucrose gradient fractions. Wild type (WT) *E. coli* and *ybeZYX-lnt* operon gene deletion mutants sucrose gradient fractions were collected and rRNA was purified and loaded on the denaturing 1.5% agarose gel. 17S 3`-end specific DNA-oligo was used for the membrane hybridization (the oligo location on 17S rRNA is shown). This oligo does not bind to mature fully processed 16S rRNA. The antibiotic treatment is annotated with red. And no treatment is also shown or not indicated. The presence of 17S rRNA in the ribosome fractions are seen. The degradation products are present massively in 30S Cam treated ribosomal fractions.

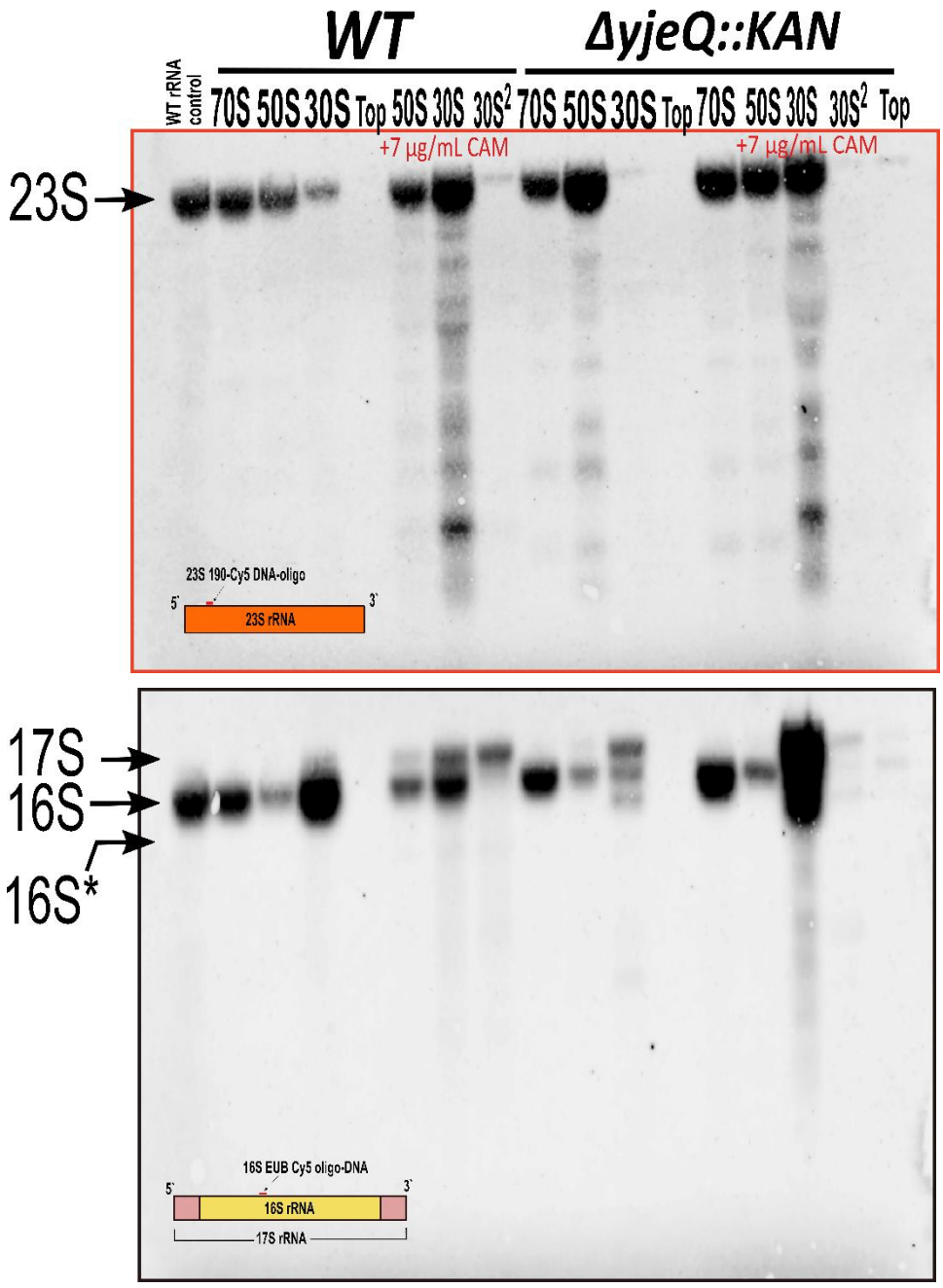

Figure S12 | Northern blot analysis of purified rRNA from sucrose gradient fractions for yjeQ deletion mutant and WT strains. Ribosome factor *yjeQ* deletion mutant cells have accumulated 16S* rRNA in their 30S fractions but did not bring any extra accumulation or 17S rRNA in the 70S fractions (both treated and not treated bacteria). 23S specific and 16S specific DNA-oligonucleotide was used for the membrane hybridization (the oligo locations are shown). The antibiotic treatment is annotated with red. And no treatment not annotated. The presence of 17S rRNA in the WT ribosome CAM treated fractions are seen. The 23S degradation products are present massively in 30S Cam treated ribosomal fractions. Seems the 23S is more effected in deletion strain.

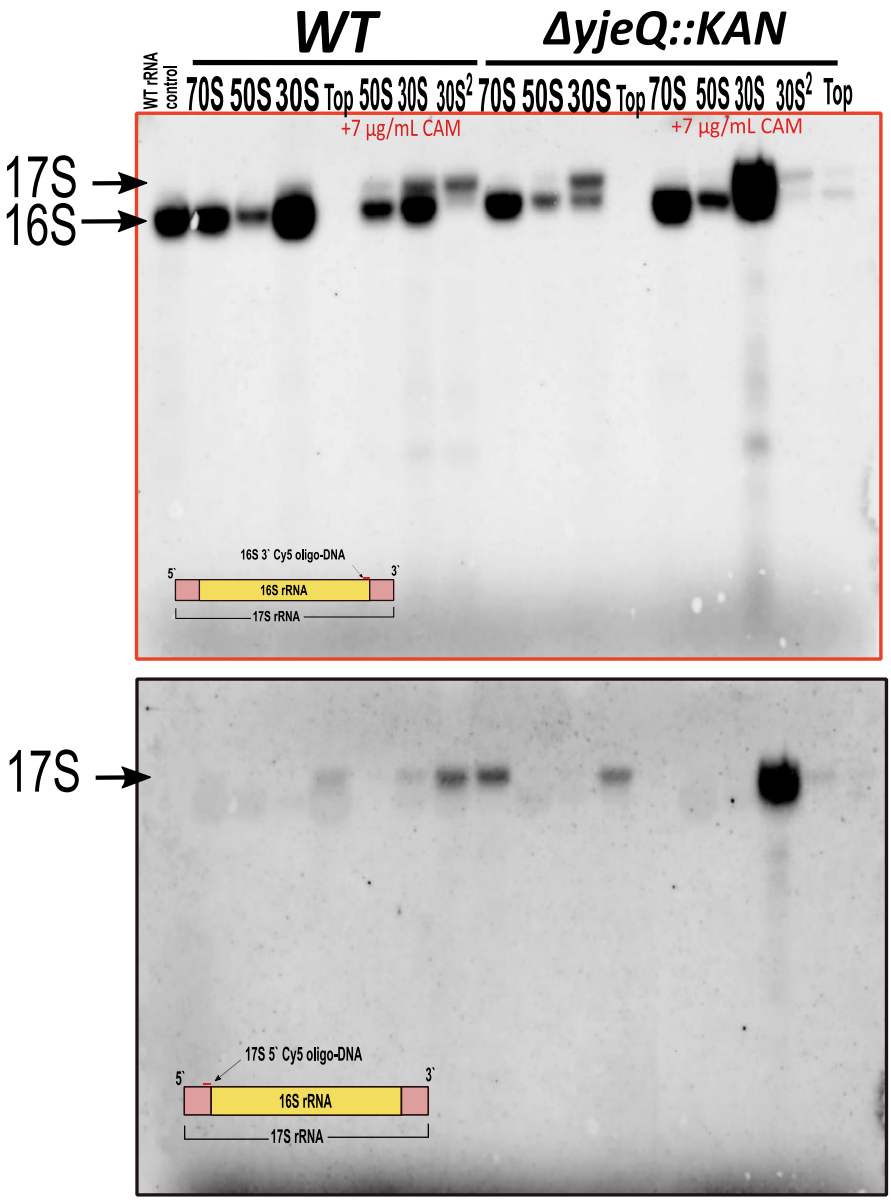

Figure S13 | Northern blot analysis of purified rRNA from sucrose gradient fractions. Ribosome factor *yjeQ* deletion mutant cause cells to have accumulated 16S* rRNA in their 30S fractions but did not bring any extra accumulation or 17S rRNA in the 70S fractions.17S 3`-end specific DNA-oligo was used for the membrane hybridization (the oligo location on 17S rRNA is shown). This oligo does not bind to mature fully processed 16S rRNA. The antibiotic treatment is annotated with red. And no treatment is also shown. The presence of 17S rRNA in the ribosome fractions are seen. The degradation products are present massively in 30S Cam treated ribosomal fractions.

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