Involvement of the putative metal efflux protein YbeX in ribosomal metabolism

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Abstract

YbeX of *Escherichia coli*, a member of the CorC protein family, is a putative Co $^{2+}$/ Mg $^{2+}$ efflux factor. Here, we describe several $\Delta\psi\beta\epsilon\Xi$ phenotypes and report an involvement of YbeX in ribosomal metabolism. *E. coli* lacking *ybeX* has a longer lag phase on outgrowth from the stationary phase. This phenotype is heterogeneous at the individual cell level and can be rescued by supplementing the growth media with magnesium. *[?]ybeX* strain is sensitive to elevated growth temperatures and to several ribosome-targeting antibiotics, which have a common ability to induce the cold shock response in *E. coli*. *[?]ybeX* cells accumulate distinct 16S rRNA degradation intermediates present in both 30S particles and 70S ribosomes. We propose that a function of YbeX is maintaining the magnesium homeostasis in the cell, which is needed for proper ribosomal assembly.

INTRODUCTION

Ribosomal biogenesis is a highly regulated process encompassing concomitant transcription, processing, degradation, modification and folding of ribosomal RNAs, equimolar synthesis, and incorporation into ribosomes of more than 50 different ribosomal proteins (Davis and Williamson, 2017). In bacteria, this is catalyzed, chaperoned and generally facilitated by dozens of dedicated proteins working in tandem in several partially overlapping and redundant pathways (Shajani*et al.* , 2011). However, due to its sheer complexity, our understanding of this process is bounded to isolated fragments of processing/folding pathways, with minimal knowledge of many individual factors' precise mechanisms of action.

It has long been known that Mg^{2+} is necessary for ribosomal assembly and translation (McCarthy *et al.* , 1962). More recently, it was discovered that intracellular free Mg^{2+} and rRNA transcription are actively co-regulated for achieving optimal ribosomal assembly and translation (Pontes*et al.* , 2016). Also, it has been shown that Mg^{2+} influx can provide an active mechanism to alleviate ribosomal stress phenotypes, probably by stabilizing ribosomal structure (Lee *et al.* , 2019).

ybeX encodes a putative Co^{2+}/Mg^{2+} efflux protein, which is highly conserved in bacteria but poorly characterized (Kazakov *et al.* , 2003; Anantharaman and Aravind, 2003). In the genome of *E. coli* , it is located in the *ybeZYX-Int* operon (**Fig. 1A**), transcripts of which have not been fully mapped. The *lnt* gene, which encodes an essential inner membrane protein, is predicted to be under the control of the minor heat shock sigma factor σ^{24}(RpoE) (Keseler *et al.* , 2013), while transcription of*ybeY* , *ybeZ* and *ybeX* is regulated by the primary heat shock sigma factor σ^{32} (RpoH) (Nonaka *et al.* , 2006). In low-magnesium conditions, the levels of YbeX (but not of YbeY and YbeZ) mRNA and protein are about two-fold reduced, consistently with its proposed role in Mg^{+2} efflux (Caglar *et al.* , 2017).

The most-studied member of the *ybeZYX-lnt* operon is the*ybeY* , whose importance in ribosomal metabolism is beyond dispute, while the precise mode of action of YbeY remains unclear (Davies*et al.* , 2010). The

YbeY is, by sequence homology and structural studies, a zinc-dependent RNA endonuclease. YbeY is universally conserved over the three domains of life, has very strong, albeit heterogeneous, phenotypes in every organism that has been looked into, and it has been shown by genetical methods to be required for the correct processing of the 3' end of 16S rRNA (Liao *et al.* , 2021). Moreover, YbeY mutants have been shown to be defective in translation and accumulate defective ribosomes in several bacterial species, mitochondria and chloroplasts (Liu *et al.* , 2015; Liao *et al.* , 2021; D'Souza *et al.* , 2021). And yet, in the purified form, its RNase activity seems to be limited to short RNA oligonucleotides (Jacob *et al.* , 2013; Babu *et al.* , 2020), while *in vitro* processing of the 16S rRNA 3'-end can be achieved without it (Smith *et al.* , 2018).

The *ybeZ* gene is located upstream of *ybeY* , having four nucleotides overlap. *ybeZ* encodes a phosphate starvation-regulated PhoH subfamily protein with the NTP hydrolase domain (Kim *et al.* , 1993). YbeZ has phosphatase activity and is a putative RNA helicase through sequence homology (Kazakov *et al.* , 2003; Andrews and Patrick, 2022). A physical interaction between YbeY and YbeZ was suggested based on bacterial two-hybrid system experiments in *E. coli* (Vercruysse *et al.* , 2016). Their interaction has been biochemically verified in *Pseudomonas aeruginosa* (Xia*et al.* , 2020).

Here we characterise the growth and ribosomal homeostasis phenotypes of the deletion of *ybeX* in *Escherichia coli* .

RESULTS

Deletion of E. coli ybeX leads to heat sensitivity and longer outgrowth from the stationary phase

ybeX is a part of the RpoH (heat response) regulon (Nonaka*et al.* , 2006). We tested by a spot assay the effect of elevated growth temperature on the *[?]ybeX* strain from the Keio collection (Baba *et al.* , 2006), compared to the isogenic BW25113. After overnight growth in the LB liquid medium, serial dilutions of the culture were spotted on LB agar plates and incubated at 20°C, 37°C or 42°C overnight. Disruption of *ybeX* hindered growth at 42°C but not at 20°C (**Fig. 1B** , **Fig. S1a**). For verification,*ybeX* deletion was reintroduced in two strain backgrounds, MG1655 and BW25113. We verified the deletion of *ybeX* and the presence of kanamycin resistance cassette by PCR analysis (**Fig. S1b, c**). Heat sensitivity occurred in both newly constructed *[?]ybeX* strains (**Fig. S1d).** This demonstrates that the observed phenotype is*ybeX* -inflicted. We used the *ybeX* deletion strain of the Keio collection in further studies.

Next, we assessed whether the lack of the YbeX protein caused heat sensitivity. Alternatively, secondary effects of the chromosomal deletion might be responsible for this phenotype. We reintroduced*ybeX* on a single-copy TranBac library plasmid (Otsuka *et al.* , 2015) and found the leaky YbeX expression in the absence of the inducer (isopropyl-β-D-1-thiogalactopyranoside; IPTG) was sufficient to rescue the heat sensitivity of the *[?]ybeX* mutant. The empty vector (pEmpty) and TranBac plasmids carrying *ybeY* or *ybeZ* had no effect on the growth (**Fig. 1B**). Thus, the heat sensitivity of the *[?]ybeX* strain was caused by the absence of the YbeX protein, rather than through polar effects on neighbouring genes.

To find which growth phase is affected by the *ybeX* deletion, we monitored bacterial cultures in liquid LB medium at 37°C on a 96-well plate reader. We did not notice differences between the growth of WT and*[?]ybeX* strains when cultures were started from freshly grown single colonies (data not shown). When cultures were inoculated with bacteria from the stationary phase overnight cultures, the *[?]ybeX*mutant had a much longer lag phase (300-350 min.) compared to the WT (100-150 min.; **Fig. 1C** , **Fig. S2a**). Both strains reached the same optical density in the stationary phase. A similar number of colonies after dilution and plating WT and *[?]ybeX*(**Fig. 1B**) indicates that the delay of the visible growth of the$\Delta\psi\beta\epsilon\Xi$ mutant is not caused by decreased survival in the stationary phase but reflects later regrowth of the same number of live bacteria. Expression of *ybeX* from a single-copy plasmid abolished the prolonged lag phase completely. In contrast, complementation with the plasmids carrying either *ybeY* , *ybeZ* or *lnt* had no effect confirming that lack of the YbeX protein is causing the delay of regrowth, while further excluding the polar effect as a cause of the$\Delta\psi\beta\epsilon\Xi$ phenotype (**Fig. 1C; Fig. S2b**).

To investigate whether the longer lag phase of $\Delta\psi\beta\epsilon\Xi$ strain is due to lower metabolic activity in the mutant

cells, we used the alamarBlue reagent, a quantitative indicator of the oxidation-reduction potential of cell membranes, as a proxy for metabolic activity (Rampersad, 2012). In a negative control experiment conducted in PBS buffer lacking the nutrients necessary for the resumption of growth, both strains show similarly low alamarBlue signal, indicating similar levels of metabolic activity (the superimposed black lines in**Fig. S2c**). When diluted into fresh LB medium, the alamarBlue signal immediately starts to increase for both strains, indicating activation of similar levels of cellular metabolism (**Fig. S2d, e**). While the initial rate of increase in the alamarBlue signal, and by implication the cellular metabolism levels, are equal for WT and$\Delta\psi\beta\epsilon\Xi$ cells, after about 100 minutes, the WT acquires a still faster rate of signal increase, while the $\Delta\psi\beta\epsilon\Xi$ cells continue as before for about 200 more minutes, before the rate of their signal growth increases to WT levels (**Fig. S2d**). As shown by the OD_{600} measurements (**Fig. S2a**), for both the WT and the $\Delta\psi\beta\epsilon\Xi$ cells, this phase shift in redox power is accompanied by the start of cell divisions (**Fig. S2e**). These results indicate that the longer lag phase of the *[?]ybeX* strain is not caused by lower levels of metabolic activity in the $\Delta\psi\beta\epsilon\Xi$cells whilst they are preparing for the resumption of cell divisions. Nor is it caused by a later onset of said metabolic activity.

The delayed outgrowth of [?]ybeX is heterogeneous at the individual cell level

When streaking out mutant strains from glycerol stocks and overnight grown stationary phase cultures, we noticed that while the *[?]ybeZ*strain produces wild-type-like colonies and *[?]ybeY* strain has uniformly small colonies, the *[?]ybeX* strain produces heterogeneous colonies. Re-streaking of small and large *[?]ybeX* colonies resulted in well-grown large second-generation colonies, indicating that the heterogeneous phenotype is not caused by a genetic mutation (data not shown). We also tested whether the colony heterogeneity is caused by freeze-thawing in the glycerol mix by growing the *[?]ybeX* and wild-type cells in liquid media into stationary phase and then plating the cells directly onto LB agar plates. Again, while wild-type cells exhibited uniform colonies, the *[?]ybeX* strain gave heterogeneous colony growth (**Fig. 2A**). Thus, it is likely that the growth heterogeneity of *[?]ybeX* depends on the heterogeneity of the initial physiological states of individual stationary cells.

We quantified colony radiuses of wild-type and *[?]ybeX* strains grown in LB and MOPS minimal media supplemented with 0.3% glucose using AutocellSeg (Khan *et al.* , 2018). *[?]ybeX* cells tend to form smaller colonies than wild-type cells when grown in LB and MOPS media (**Fig. 2A**). *[?]ybeX* colonies are heterogeneous when grown in LB medium (**Fig. 2B**), while the colony radiuses are homogeneous when *[?]ybeX* cells are grown in MOPS liquid medium (**Fig. 2C**).

We then asked how heat shock affects cell growth and heterogeneity. Overnight cultures were diluted and plated on LB agar plates following 16-18 hours of incubation at 37°C or 42°C (**Fig. S3a**). We observed fewer *[?]ybeX* colonies at 42degC ($p < 0.0001$ and $p = 0.02$ for LB and MOPS, respectively; **Fig. 2D**).

We inspected the colony growth of *[?]ybeX::kan* (*ybeX* single deletion strain in BW25113 background constructed via lambda red recombination) and *[?]ybeX/kan-* (the kanamycin cassette removed from the inhouse constructed *[?]ybeX::kan*) cells at 37degC for 24 and 48 hours (**Fig. S3b, c**). No significant differences were observed for *[?]ybeX::kan* and *[?]ybeX/kan-* . Furthermore, although the observed tiny colonies of *[?]ybeX* were increasing in size over time, they consistently remained smaller than WT-like*[?]ybeX* colonies (**Fig. S3b, c**).

To better understand the nature of the observed lag phase phenotype at the individual cell level, we quantified colony radiuses of the*[?]ybeX* and the WT cells at 37degC and 42degC using cells pre-grown for 16-18 hours in liquid LB or MOPs minimal media prior to plating. Inspection of four independent stationary phase outgrowth experiments showed, in accordance with our previous observations, that at both temperatures WT cells tend to form larger colonies, while the colony radiuses of *[?]ybeX* cells are heterogeneous and possibly dimorphic. These intuitions were formalized by jointly modelling means and standard deviations of colony radiuses and, in a separate model, the colony radiuses as mixtures of two normal distributions (see Materials and Methods for details). The estimated mean colony radiuses are smaller in the ybeX strain by about 1/3 (the difference, in arbitrary units, at 37degC is 3.58 [95% CI 2.89, 4.24] and at 42degC is 3.72

[3.01, 4.42]), and the *[?]ybeX* colony radiuses have a larger standard deviation (the difference in 37degC is 0.67 [0.36, 1.04] and at 42degC is 0.91 [0.53, 1.34]). Interestingly, modelling the colony radiuses as emanating from two distinct gaussian populations resulted in a superior out-of-sample fit, as assessed by leave-one-out-cross validation (data not shown), supporting the conjecture that *[?]ybeX*cells grow in at least two distinct regimes, one of which is similar to WT growth, while another results in up to two-fold smaller colonies (WT vs. *[?]ybeX* difference in 37degC: μ_1 (estimate for the mean of the first gaussian): 1.73 [0.47, 2.93], μ_2: 5.58 [4.71, 6.39], and in 42°C: μ_1:1.39 [0.06, 2.81], μ_2: 5.92 [4.99, 6.79]).

The [?]ybeX strain is sensitive to ribosome-targeting antibiotics

ybeX disruption has been reported to cause cell death in the presence of chloramphenicol (Smith *et al.* , 2007). We therefore explored the effects of various antibiotics on the *[?]ybeX* cells. First, we determined the minimal inhibitory concentrations (MICs) in LB for the *WT* and the *[?]ybeX* strains (see Materials and Methods). The MICs were two times lower for *[?]ybeX* in the presence of fusidic acid, clindamycin, chloramphenicol, tetracycline and erythromycin (**Table S1**). These structurally unrelated ribosome-targeting antibiotics have been shown to induce cold-shock proteins or block the induction of heat-shock proteins (VanBogelen and Neidhardt, 1990; Cruz-Loya *et al.* , 2019). We further inspected the effects of these antibiotics using the dot spot assay described above, except that the LB agar plates were supplemented with sub-inhibitory concentrations of indicated antibiotics (see Materials and Methods). The *[?]ybeX* strain exhibited severe sensitivity to sub-lethal concentrations of all of these antibiotics (**Fig. 3A**). Expressing the YbeX protein from a single-copy plasmid in the absence of the inducer completely rescued the antibiotic sensitivity (**Fig. 3B**). In contrast, protein synthesis-targeting antibiotics, for which we do not have evidence that they affect the cold shock response (amikacin, streptomycin, kanamycin, tobramycin and mupirocin), was founded not to have an effect. In addition, the transcriptional inhibitor rifampicin revealed no effect on the$\Delta\psi\beta\epsilon\Xi$ strain compared to the WT (**Fig. 3B, Fig. S4b**). We also measured the MICs in the MHB cation-adjusted media. The MICs for wild type and *ybeZ* , and *ybeX* deletion strains remained the same, while *ybeY* deletion strain showed lower MICs for all tested antibiotics (data not shown).

We also tested the survival of two isogenic wild-type strains, MG1655 and BW25113 and the corresponding deletion strains*[?]ybeX::kan*^{MG} and*[?]ybeX::kan*^{BW} under sub-inhibitory antibiotic concentrations. Both genetic backgrounds exhibited similar antibiotic sensitivities, and removal of the kanamycin resistance cassette (in strains *[?]ybeX/-kan*^{MG} and*[?]ybeX/-kan*^{BW}) had no effect (**Fig. S4**). In contrast, ectopic expression of *ybeX* in the absence of an inducer abolished the antibiotics sensitivity (**Fig. 3B**).

The antibiotic sensitivity of [?]ybeX depends on the growth history of cells

Our finding that while the *[?]ybeX* cells have a lengthened lag phase during outgrowth from the stationary phase, they appear to retain similar levels of metabolic activity during this lag phase to the WT cells, as well as similar exponential growth rate (**Fig. 1D**), led us to hypothesize that any cellular defects conferred by the lack of YbeX may accumulate during the late growth, preceding entry into the stationary phase and/or in the stationary phase itself. Such a stochastic process could lead to the observed single-cell level growth heterogeneity (**Fig. 2**). Accordingly, we assayed whether the phenotypes of *[?]ybeZ* , *[?]ybeY,* and *[?]ybeX* depend on the growth phase where the cells originate. We surmised that if the*[?]ybeX* phenotype is caused by a gradual accumulation of harm, then cells that have been given ample time to accumulate such harm, should exhibit a stronger phenotype than cells with only a few divisions.

First, we tested the antibiotic sensitivity phenotype. In this experimental setup, we start by growing a single bacterial colony for 12 hours into the early stationary phase (**Fig. 4A**). Then, the experiment is divided into two. In the first arm, to assay the antibiotic sensitivity during outgrowth, stationary liquid cultures are directly dot spotted into agar plates containing sub-inhibitory concentrations of antibiotics. In the second arm, to assay the antibiotic sensitivity of exponentially growing cells, the same stationary cultures are first diluted a hundred-fold into fresh liquid media and grown at 37°C for four to five cell divisions until OD_{600} reaches 0.2-0.4, after which they are dot spotted.

The *[?]ybeX* strain exhibited very strong chloramphenicol and erythromycin sensitivity in cells originating

from the early stationary phase but no sensitivity to Rifampin (**Fig. 4B**). In contrast, the *[?]ybeX* cells plated on the antibiotic after only a few rounds of the division had WT-like sensitivity to all tested antibiotics. In comparison, *[?]ybeZ* cultures had similar intermediate levels of sensitivity to chloramphenicol, regardless of the growth history of cells, while they are not sensitive to erythromycin, rifampicin, and tetracycline (**Fig. 4B**). Exponentially growing *[?]ybeZ*cells in MOPS minimal medium, supplemented with 0.3% glucose as the carbon source, also exhibited sensitivity to chloramphenicol (**Figure S4a**). *[?]ybeY* cells had a very strong sensitivity to all tested antibiotics under both growth conditions. This is not surprising, considering its strong growth phenotype.

Testing the culture growth in liquid media, after diluting the culture directly from the early stationary phase, again showed a lengthened lag phase for *[?]ybeX* but not for *[?]ybeZ* , while the exponential growth rates of both *[?]ybeX* and *[?]ybeZ* were very similar to WT (**Fig. 4C**). The *[?]ybeY* strain behaves similarly in both experiments, exhibiting a reduced exponential growth rate and reaching a lower maximal cell density. In contrast, when the cells are outgrown from exponential phase cultures, the WT, *[?]ybeZ* and *[?]ybeX*strains grow equally well, with no visible lag phase, while the*[?]ybeY* strain has a reduced growth rate and a lower growth end-point, as expected (**Fig. 4D**).

[?]ybeX cells accumulate rRNA fragments

As *ybeX* is located in the same operon with *ybeY,* whose role is implied in ribosome assembly, we assessed the rRNA profiles of WT Keio and *[?]ybeZ* , *[?]ybeY* , and *[?]ybeX* strains by formaldehyde denaturing agarose gel electrophoresis of total cellular RNA. In exponentially growing *[?]ybeY* cells, we saw a substantial accumulation of immature 16S rRNA (17S rRNA), while *[?]ybeX* and*[?]ybeZ* cells had comparable levels of 17S rRNA to wild-type (**Fig. 5A**). *[?]ybeY* cells also accumulate a faster-migrating 16S rRNA species, labelled as 16S* (**Figure 5A;**see also (Davies *et al.* , 2010)). When we assessed the RNA extracted from stationary phase cultures in *[?]ybeX* cells we observed a major RNA fragment of about a thousand nucleotides (**Fig 5A**). This fragment was not present in material obtained from exponentially grown *[?]ybeX* cells. The wild type, the*[?]ybeZ* and the *[?]ybeY* cells exhibited no such fragments in either stationary or exponential cells.

We used a more sensitive assay, the Northern blotting, on total RNA.**Fig. 5B** shows, for *[?]ybeX* lysates, a wide spectrum of 16S rRNA intermediates ranging from 500 nt (our lower detection limit) to almost full length 16S rRNA. Note that due to apparent cross-binding of our 16S-targeting probe to the 23S rRNA, we also see the 23S rRNAs as distinct bands in the gel, but importantly there are no degradation fragments between the full length 23S rRNA and 17S rRNA in any of the strains. Also, the 17S pre-rRNA is present for *[?]ybeX* and*[?]ybeY* . Interestingly, *[?]ybeX* does not contain the 16S* rRNA species, which is present in *[?]ybeY* (but not *[?]ybeX*). Except for the 16S* rRNA of *[?]ybeY* , the WT, *[?]ybeZ* and *[?]ybeY*lanes lack degradation intermediates. Thus, the *[?]ybeX* cells contain a unique and disparate mixture of 16S rRNA degradation intermediates.

The [?]ybeX strain accumulates distinct rRNA species already during the late exponential growth

As there is neither assembly nor degradation of mature ribosomes in the early stationary phase (Piir *et al.* , 2011), we conjectured that the 16S fragments observed in *[?]ybeX* cells were likely accumulating by the late exponential phase. Accordingly, we purified, from late exponential cells, ribosomal subunits by sucrose gradient fractionation and analyzed the rRNA composition of the 70S ribosomes, as well as 50S and 30S subunits by Northern blotting. In these experiments we used probes specific for both ends of 17S precursor, for the 16S 3' end, and for the mature 16S rRNA and 23S rRNA, allowing us to see degradation intermediates emanating from immature pre-16S rRNAs (**Fig. 6A**).

The sucrose gradient profiles for WT and *[?]ybeX* lysates are very similar, with the vast majority of ribosomal particles being in the presumably active 70S ribosome fraction and the small free subunit fractions exhibiting no obvious abnormalities (**Fig. 6B**). The Northern blots revealed 17S precursor rRNAs in the 30S fractions of both the WT and the *[?]ybeX* strain, likely due to active ribosomal synthesis in both strains (**Fig. 6C, E**). In addition, in the*[?]ybeX* strain the mature 16S rRNA species is substantially reduced in the 30S fraction,

so that the 17S to 16S ratio is clearly shifted in relation to WT. Thus, in the *[?]ybeX* strain the 30S fraction is unlikely to contain many functionally active ribosomal subunits.

In addition, there are two distinct 16S fragments, both around 1 kb long (truncated ribosomal RNA species denoted as "trunc."), in the ribosomal fractions originating from the *[?]ybeX* cells. Firstly, there is a major 5' end-truncated 16S rRNA fragment which is present in all ribosomal fractions, including the 70S ribosomes (**Fig. 6C, E**). In the 30S fraction this fragment is produced already from the 17S pre-rRNA (**Fig. 6F**), but the same fragment in the 70S ribosomes is not of this origin, presumably originating from full length mature 16S rRNA inside the 70S particles (**Fig. 6E**). Its presence in the 30S fraction is more pronounced than that of the 17S pre-rRNA, indicating that most of the pre-30S particles are inactive and degradation-bound in late exponential phase $\Delta\psi\beta\epsilon\Xi$ cultures. Secondly, there is a slightly larger 3' end-truncated 16S RNA fragment (**Fig. 6E**), which is present in the 30S fraction only (**Fig. 6G**). This fragment also originates from the 17S precursor particles. In contrast, 23S rRNA specific probe reveals several relatively minor differences in degradation patterns between WT and$\Delta\psi\beta\epsilon\Xi$ strains (**Fig. 6D**).

Taken together, these results indicate that in the late exponential phase the majority of free 30S $\Delta\psi\beta\epsilon\Xi$ strain is in the process of being degraded. Moreover, the degradation fragments captured by the pre-16S rRNA specific probes strongly suggest that in the $\Delta\psi\beta\epsilon\Xi$strain both pre-ribosomes (in the 30S fraction) and mature ribosomes (in the 70S fraction) are susceptible to degradation. While a majority of pre-ribosomes in the 30S fraction appear as the 1000-nt rRNA fragment (**Fig. 6E, F**), a minority of mature 70S is present as the 1000-nt fragment.

[?]ybeX perturbs ribosomal assembly through a separate mechanism from chloramphenicol

The strong sensitivity of *[?]ybeX* cells to chloramphenicol (CAM) treatment prompted us to investigate the chloramphenicol phenotype further. CAM is a well-studied inhibitor of protein synthesis that binds to the large ribosomal subunit, inhibiting peptidyl transfer (Wilson, 2014). The effect of chloramphenicol on cell growth is at least partially mediated by the imbalanced synthesis of r-proteins, which results in the accumulation of partially assembled and misassembled ribosomal subunits (Siibak *et al.* , 2009).

We tested the effect of sub-inhibitory concentrations of CAM on the ribosomes using sucrose gradient fractionation and northern blotting. Overnight-grown cells were diluted in liquid LB medium and grown until cells reached mid-exponential growth (OD_{600}=0.3). The CAM treatment took place for 2 hours. The cells were also grown without CAM for 2 hours as a control (**Fig. 7A**).

While the CAM particles were formed in both wild type and *[?]ybeX*strains (**Fig. 7B**), we failed to observe any aberrant rRNA species for the WT strain (**Fig. 7C**), while the accumulation of the distinct rRNA species appeared in *[?]ybeX* cells repeatedly (**Fig. 7D**). Thus, the mechanism that leads to the degradation of pre-rRNA in 30S particles in *[?]ybeX* cells seems to be different from that of the imbalanced protein synthesis caused by CAM. The CAM action mechanism also appears to stabilize *[?]ybeX* 30S particles, while the pre-16S rRNA degradation intermediate is present in both 70S and 50S fractions. We believe its presence in the 50S to be due to cross-contamination from the 70S fraction. Interestingly, while WT CAM 70S particles contain a good measure of 17S pre-rRNA (which is absent in WT non-treated cultures), the *[?]ybeX* CAM 70S particles, although containing the degradation intermediate, do not have this pre-16S rRNA species. These results suggest that the perturbation of assembly by CAM and by *[?]ybeX* go by separate and at least partially independent mechanisms.

The [?]ybeX phenotype can be suppressed by $MgCl_2$

As YbeX has been implicated in Mg^{+2} efflux (Gibson*et al.* , 1991), we tested whether supplementing growth media with magnesium chloride affects the *[?]ybeX* phenotype. First, we compared the growth of WT and *[?]ybeX* in LB medium with and without magnesium supplementation (**Fig. 8A, Fig. S5a**). When the LB medium was supplemented with 10 mM $MgCl_2$, the antibiotic sensitivity and heat shock phenotypes of *[?]ybeX* disappeared (**Fig. 8A**). To test whether the effect is media-dependent, we used the SOB medium, which contains a high 10 mM concentration of $MgCl_2$. Again, the phenotypes of *[?]ybeX* disappeared (**Fig.**

S5a,b). Thus, excess magnesium in the growth media, either LB or SOB, fully rescues the outgrowth growth phenotypes of the*[?]ybeX* cells.

To test whether magnesium-deficient-rich media could increase the severity of the growth phenotype, we used the peptide-based medium (PBM), a rich, magnesium-limited, buffered, complex growth medium (Christensen *et al.* , 2017). PBM is advantageous because it is free of any cell extract, which is the primary source of magnesium in almost all complex media (Li *et al.* , 2020). To avoid diauxic inhibition, we modified it to contain casamino acids instead of glucose as the carbon source (see Materials and Methods). *[?]ybeX* cells had longer lag times on outgrowth compared to when grown in PBM (**Fig. S5c**), while wild-type cells grown in LB or PBM didn't differ. When PBM is not supplemented with $MgCl_2$, the heat shock phenotype is more substantial than in LB medium (**Fig. 8B** , compare **Fig. S5d with Fig. S5e**). Supplementing PBM with 50 μM and 100 μM $MgCl_2$ partially suppresses the phenotype, first at 37°C and then at 42°C, and supplementation with 200 μM $MgCl_2$ completely suppresses it under both temperature conditions (**Fig. 8B**). Therefore, we have a potentially sensitive regulatable system for driving the growth phenotype of the*[?]ybeX* strain.

To test the sensitivity and robustness of such an experimental system, we did a liquid medium growth experiment in defined MOPS minimal medium, with glucose as the carbon source. Unlike with the PBM, in the MOPS medium, we can precisely control the magnesium levels by adding $MgCl_2$ from 10 μM to 525 μM (the "normal" optimal level for this medium; (Neidhardt *et al.* , 1974)). When the WT cells grew into an overnight stationary phase in different Mg^{2+}-depleted MOPS media, there was no Mg^{2+} supplementation effect for the outgrowth lag phase duration (**Fig. 8C** , the left panel). As expected, there was no effect on the actual growth rate after the lag phase. Under the same conditions, the Mg-supplementation effect on *[?]ybeX* lag phase was very different (**Fig. 8C** , the right panel). There appears a threshold effect, whereby Mg^{2+}-supplementations by 50 μM and less produce a slight gradual shortening of the lag phase from around 400 minutes to 350 minutes, while supplementation with 75 μM $MgCl_2$ suddenly shifts the lag time to about 200 minutes, after which additional magnesium has little effect on the duration of the lag phase.

Δψβϵξ πηϵνοτψπϵ αππϵαρς δυριν γ τηϵ τρανσιτιον ιντο τηϵ στατιοναρψ γροωτη πηασϵ

Our ability to control the *[?]ybeX* phenotype by Mg^{2+} allows us to pinpoint the growth-phase dependence of the *[?]ybeX* phenotype more precisely. Therefore, in the next experiment, we start by growing the cells under high magnesium, where*[?]ybeX* phenotype does not occur, and then do the outgrowth under low magnesium to look for the gradual appearance of the phenotype. We first grew the cultures overnight into the stationary phase, starting from a single colony in MOPS minimal medium supplemented with 10 mM $MgCl_2$. This protocol was used to suppress the*[?]ybeX* phenotype. These cells were then pelleted and washed thrice in MOPS minimal media lacking Mg^{2+}. Then after the regrowth assay was set up by suspending the cells in the MOPS minimal medium containing 10 μM $MgCl_2$ so that the starting optical density of the regrowth culture would be 0.05 OD_{600} units (**Fig. 9A**). Here, we expected to see the gradual onset of the *[?]ybeX* phenotype.

As expected, there is now no difference in the duration of the pre-outgrowth lag phase between the strains. During outgrowth, the exponential growth rates were the same for wild-type and *[?]ybeX*(**Fig. 9B**). There is also no difference in growth during the gradual onset of the stationary phase, as the *[?]ybeX* cells end up in a very similar end-of-growth plateau, which holds for a wide range of Mg^{2+} concentrations in the medium (**Fig. 9C**). To look for the emerging *[?]ybeX* phenotype, we dot spotted samples from the outgrowth cultures at designated time points (as shown in**Fig. 9B**) onto both LB agar and the R2A agar plates at 37°C and onto LB agar at 42°C. This experiment shows that during the exponential growth phase (up until 4 hrs time point), there is no growth phenotype for the *[?]ybeX* strain (**Fig. 9D)** . In contrast, during the transition into the stationary phase, as measured in the 5.5h time point, there is an apparent growth effect of the *[?]ybeX* strain at both growth media, which is more pronounced at 42degC heat shock conditions. Similarly, the relative sensitivity of the *[?]ybeX*strain vs. the WT strain to the antibiotics Tetracyclin, Erythromycin and Chloramphenicol appears only at the 5.5h time point, the effect being much stronger than for the previous outgrowth assay done without the antibiotics (**Fig. 9E**). From these experiments we

conclude that the growth phenotype of the *[?]ybeX* strain, and its antibiotic sensitivity, appear only at the transition between the exponential and stationary growth phases.

DISCUSSION

This work shows that the putative Co^{2+}/Mg^{2+} efflux protein YbeX is functionally involved in ribosome metabolism in *Escherichia coli* . For a possible mechanism that is consistent with experimental results, we propose that during growth without *ybeX,* there is an accumulation of harm in the late-exponential growth phase involving pre-17S rRNA and 16S rRNA partial degradation products (**Fig. 6C-G**), which necessitates a longer lag phase upon outgrowth in a fresh medium. During this prolonged lag phase, the *[?]ybeX* cells are metabolically active (**Fig. 1D**) and would be busy cleaning up the inactive and/or partially degraded ribosomal particles before new ribosome synthesis and subsequent cell division can commence. An inability to do so properly in the absence of the YbeX protein then leads to the growth phenotype and antibiotic sensitivity during the shift from exponential growth to stationary phase (**Fig. 9**). Intriguingly, although the late-exponential phase *[?]ybeX* cells accumulate rRNA degradation products, to some extent, even in the 70S fraction (**Fig. 6C, E**), they have WT-like sucrose gradient profiles (**Fig. 6B**), indicating no accumulation of significant ribosome-like particles. In addition, although the *[?]ybeX* cells have a clear growth phenotype, manifested in a lengthened outgrowth lag phase and in sensitivity to antibiotics, the exponential growth rate of the *[?]ybeX* cells is indistinguishable from WT, as are the growth end-points (**Fig. 1C, Fig. 8C, Fig. 9B**).

We find that the growth phenotype of *[?]ybeX* is Mg^{2+}-dependent, being present in Mg^{2+}-limiting growth conditions (**Fig. 8**). This result is consistent with its proposed role in Mg^{2+} efflux in *Salmonella typhimurium* (Gibson et al. 1991). However, to mechanistically tie the YbeX protein with Mg^{2+} metabolism, requires considerably more experimental work. Currently, the totality of evidence is highly suggestive of the role of YbeX in regulating Magnesium homeostasis, but the exact mechanism should still be considered as open.

What could be the mechanism of action of the YbeX protein on the ribosome? Unlike its neighbouring gene products, the YbeY and the YbeZ, there is no evidence that YbeX binds to the ribosome or to any ribosome-associated protein. Nonetheless, at this stage, we cannot exclude the possibility of a direct action of the YbeX on the ribosome. The *ybeX /corC* gene was initially recovered in *S. typhimurium* in a screen for resistance to cobalt and proposed to contribute, possibly as a co-effector of the trans-membrane metal transport protein CorA, to the efflux of divalent cations (Gibson*et al.* , 1991). As yet, there is no mechanistic function ascribed to YbeX, and while Mg^{2+} influx is generally well-studied, its efflux is poorly understood in bacteria (Armitano*et al.* , 2016). Essentially, YbeX is a cytoplasmic protein (Sueki*et al.* , 2020), for which we have indirect evidence that it might be somehow involved in Mg^{2+}-efflux. Our finding that the growth phenotype of the *[?]ybeX* strain needs low extracellular Mg^{2+} is consistent with the role of YbeX in Mg^{2+} efflux, as Mg^{2+} efflux is inhibited at low extracellular magnesium (Nelson and Kennedy, 1971) and can be activated by adding 1mM $MgCl_2$ to the growth medium for *S. typhimurium* (Gibson *et al.* , 1991). Our results suggest that this activation, which occurs by an unknown mechanism, may involve a discontinuous switch, occurring somewhere between 50 µM and 75 µM $MgCl_2$ concentration (**Fig. 8C**). When thinking about the activation of Mg^{2+} efflux by increasing extracellular Mg^{2+} concentrations, we also need to consider the effect of low extracellular Mg^{2+} on cellular physiology. The extracellular Mg^{2+} acts as a counterion to neutralize the phosphate groups of outer-membrane lipopolysaccharides, and it binds to many membrane proteins, stabilizing their structures (Groisman and Chan, 2021). Accordingly, a lack of extracellular Mg^{2+} leads to permeabilization of the outer membrane, including for hydrophobic antibiotics like Erythromycin and Rifampin (Vaara, 1992).

YbeX has been genetically connected to translation, as *E. coli*cells that rely for growth on an artificial ribosome variant, where the subunits are covalently tethered by fused rRNAs, need for faster growth a nonsense mutation in the *ybeX* gene, together with a missense mutation in the *rpsA* (Orelle *et al.* , 2015). A mechanism of action could be that the suppressed Mg^{2+} efflux in the absence of *ybeX* leads to increased cytoplasmic Mg^{2+} concentration, stabilizing the artificial ribosomes and thus activating them for protein

synthesis.

On the other hand, we have found that a very high, sublethal, Mg^{2+} concentration (200 mM) in LB liquid media gradually leads to cell death and the emergence of aggregates, giving the *[?]ybeX* strain a survival advantage over the WT, which increases to an order of magnitude during 6 hours of incubation (**Fig. S6**). While this positive effect can only be seen under very high Mg^{2+} concentrations (between 200 mM and 100 mM, data not shown), it is the opposite of what one expects to see when growing an Mg^{2+}-efflux deficient strain in extremely high extracellular $MgCl_2$. Clearly, the mechanistic role of the YbeX in $MgCl_2$ homeostasis awaits further clarification.

We currently favor the provisional model whereby the effect of*ybeX* deletion on ribosomal metabolism is indirect, happening through an increased concentration of intracellular Mg^{2+}. According to this model, the YbeX-promoted Mg^{2+} efflux is needed in the late exponential phase, when cell growth rates begin to fall, and ribosomes are degraded (Piir*et al.* , 2011), releasing some of the ribosome-bound, as well as NTP-bound, Mg^{2+} into the free Mg^{2+}pool. As both very low and very high Mg^{2+}concentrations are detrimental to cells, mainly through translation, the intracellular free Mg^{2+} is tightly controlled between 1 mM and 5 mM (Akanuma, 2021) and actively regulated Mg^{2+} efflux can be an integral part of metal homeostasis management in bacteria (Wendel *et al.* , 2022).*In vitro* translation is very sensitive to increased Mg^{2+} concentration, already being >95% inhibited at 6 mM $MgCl_2$ (Borg and Ehrenberg, 2015). Intriguingly, and in accordance with the role of YbeX in maintaining Mg^{2+}-homeostasis, we found that expression of the*ybeX* from a high-copy plasmid is toxic to both WT and*[?]ybeX* cells, even in the absence of an inducer.

In conclusion, our work emphasizes that the role of magnesium homeostasis in ribosomal metabolism should become an increasingly fertile field of study.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Media

Genotypes of the bacterial strains, plasmid descriptions, and sequences of primers used in this study are listed in Tables S2-S3. Bacteria were grown in Difco™ LB Broth (BD brand #240230 consisting of Tryptone 10 g/L, Yeast Extract 5 g/L, Sodium Chloride 5 g/L). LB agar plates were prepared from Difco™ LB Agar (BD brand #240110). The growth media was supplemented with an appropriate amount of antibiotics (100 µg/mL ampicillin, 25 µg/mL chloramphenicol, 50 µg/mL kanamycin, 12.5 µg/mL tetracycline) when necessary for the selection of strains and maintenance of plasmids.

Keio collection deletion strains, including *[?]ybeX* , *[?]ybeY* , and *[?]ybeZ* , and *Escherichia coli* wild-type BW25113 strains were used in this study (Baba *et al.* , 2006). We also reconstructed the *ybeX* single deletion strain using *E. coli* MG1655 and BW25113 via lambda red recombination (Datsenko and Wanner, 2000). The kanamycin resistance gene (kan) was removed from the bacterial chromosome using the pCP20 plasmid.

E. ϛολι ΔH5α strain was used for plasmid cloning and propagation. In addition, the TransBac library, a new *E. coli* overexpression library based on a single-copy vector, was obtained from Dr Hirotada Mori (Nara Institute of Science and Technology, Japan) as a stab stock (Otsuka *et al.* , 2015).

Conjugation of the TransBac library plasmids

Hfr strain is the donor strain that carries each TranBac library plasmid and can transfer the target plasmid by conjugation (unpublished resource by Mori). The donor strain was grown on LB agar plates supplemented with tetracycline (Tc) and 25-50 µg/mL diaminopimelic acid (DAP). *Hfr* strain requires DAP because of the deletion of the*dapA* gene. Well-grown donor and acceptor cell cultures were mixed 1:1 ratio in a 1.5 mL polypropylene tube and incubated at 37°C for 1 hour without shaking. After the appropriate time for conjugation, the cell mix was plated onto LB agar plates containing Tc (12.5 µg/mL) without DAP. The plates were incubated at 37°C overnight.

Construction of the TransBac empty (pTB-empty) plasmid

The single-copy TransBac library plasmid coding *ybeX* was purified using an in-house alkaline lysis method followed by purification via FavorPrep plasmid DNA extraction mini kit (Favorgen, Austria). The cloning site was sequenced by Sanger sequencing. The *ybeX* coding region was removed via restriction enzyme cleavage of *XmaJI* and*SfiI* (Thermo Scientific). The sticky ends were filled using Klenow fragment (Thermo Scientific), and the linear plasmid was ligated using T4 DNA ligase (Thermo Scientific) following manufacturer protocols. The ligation reaction was transformed into Inoue *E. ϛολι ΔH5α* chemical competent cells (Green and Sambrook, 2020), and the TransBac empty backbone plasmid was purified as mentioned above. The size of the plasmid DNA was determined via agarose gel electrophoresis, and the cloning site was sequenced. The plasmid was electroporated into Keio collection strains BW25113 and *[?]ybeX* .

Preparation of the Peptide Based Media (PBM)

Growth in peptide-based media (PBM) is magnesium-limited (Christensen*et al.* , 2017). The previously described PBM recipe requires adding 0.4% of glucose (4 g/L) as a carbon source. We prepared the PBM via dissolving 10g/L Peptone, 1.5% casein hydrolysate (casamino acids) and 40 mM MOPS (3-(N-morpholino) propane sulfonic acid) buffer pH 7.4. The 50x MOPS buffer stock solution contained 2M MOPS and 0.2M Tricine pH 7.4 set with concentrated KOH. We achieved extremely high cell densities, OD_{600} = 10-13, when 2x PBM were used in the presence of 1-10 mM $MgCl_2$ or $MgSO_4$.

Bacterial Spot Assay

Bacterial cell cultures were diluted to final OD_{600} = 0.125, the first dilution (10^{-1}), and then 10x serial dilutions were applied. 5µL of each dilution were spotted on LB agar plates with or without antibiotics (No AB) supplementation. The sub-inhibitory concentrations of the antibiotics were as follows; 2.5-4 µg/mL chloramphenicol, 0.5-1 µg/mL tetracycline, 20-40 µg/mL erythromycin, 50 µg/mL clindamycin, 50-100 µg/mL fusidic acid, 10-20 µg/mL mupirocin, 0.5 µg/mL tobramycin or amikacin, 2.5 µg/mL rifampicin, 2.5 µg/mL streptomycin. The plates were imaged using an Epson Expression 1680-pro scanner.

Colony Size Characterization and Quantification

Keio wild-type and *[?]ybeX* strains were grown overnight in LB or defined MOPS minimal media (Neidhardt *et al.* , 1974). Well-grown bacterial cell cultures were serially diluted and plated on LB agar plates using glass beads (Hecht Assistent, #41401004). We aimed to have approximately 100-125 colonies per plate. The plates were incubated overnight at 37°C or 42°C and scanned using EPSON Expression 1680pro scanner. The images were subjected to AutoCellSeg software (Khan*et al.* , 2018). The colonies were first picked automatically using program default settings, and then, as a second step, manual picking was applied (picking small colonies, deselecting adherent colonies, etc.). The data were analysed in the R::tidyverse package (Wickham *et al.* , 2019; R Core Team, 2022).

Growth monitoring in the 96-well plate reader

C ells were diluted in the appropriate growth media to OD_{600} = 0.55, and 10 µL of the diluted cells were transferred into 100 µL of growth media in a 96-well plate. The 96-well plate edges were filled with distilled water. The remaining 60 wells were used to monitor the growth. At least one column was always set as a sterility control. Alamar Blue reagent (BioRad, #BUF012B) was used per the manufacturer's protocol (excitation 545 nm, emission 590 nm). BioTek Synergy Mx or H1 microplate readers were used.

Sucrose gradient fractionation

E. coli strains from the Keio collection were streaked onto LB agar plates and grown overnight at 37°C. A single colony of each strain was inoculated into LB and aerated at 37°C overnight. The following morning, the culture densities were determined via spectrophotometer (Biochrom Ultrospec 7000); the cells were diluted to a final OD_{600} of 0.05-0.06 in LB medium (150-250mL) and grown until OD_{600} = 0.3-0.35. The cultures were then split into two flasks, in which the chloramphenicol treatment was carried out, while the other was grown as a control for 2 hours.

The cells were transferred into centrifugation bottles, cooled on ice and pelleted at 4000xg, at +4°C for 10 minutes. The supernatant was removed, and the cell pellet was snap-frozen in liquid nitrogen and stored at -80°C. The cells were dissolved in 1 mL of lysis buffer consisting of 25 mM Tris-HCl pH 7.9, 60 mM KCl, 60 mM NH_4Cl, 6 mM $MgCl_2$, 5% glycerol supplemented with 1mM PMSF, protease inhibitor (Roche, #04693159001) and 5mM βME added freshly to the buffer before the lysis. The cells were lysed using FastPrep homogenizer (MP Biomedicals) by three 40-second pulses at 4.0 m/s, chilling on ice for 5 min between the cycles. The beads were purchased from BioSpec Products, and 0.4 gram of 0.5mm Zirconia/Silica beads (BioSpec, #11079105z) and 0.9 gram of 0.1mm Zirconia/Silica beads (BioSpec, #11079101z) was used.

The lysate was clarified by centrifugation 16,100xg for 40 minutes at 4°C. Clarified lysates were treated with 50 units/mL DNase I (MN, #740963). The lysates were loaded onto 10-30% sucrose gradients in a buffer containing 25mM Tris-HCl pH 7.9, 100mM KCl, 10mM $MgCl_2$, supplemented with 5mM βME. The gradients were centrifugated at 20,400 rpm for 17 h at 4°C in an SW-28 Beckman Coulter rotor (ω^2t=2.8e+11). The samples from the gradient were pumped starting from the bottom through a spectrophotometer (Econo UV Monitor, BIO-RAD), which can detect A254 as a readout. The data was recorded by Data Acquisition software (DataQ Instruments) and imported into R for plotting (R Core Team, 2022).

Purification of rRNA from Ribonucleoprotein (RNP) Complexes

Ribosomes and ribosomal subunits were collected from sucrose gradients as peak fractions. The sucrose fractions were collected into 15 mL falcon tubes and diluted at least two-fold with the gradient buffer (25mM Tris-HCl pH 7.9, 100mM KCl, 10mM $MgCl_2$). Next, 2.5 vol. of 96% ethanol was added to the samples and incubated at -20°C overnight. The fractions were pelleted via centrifugation for 45 minutes at 4000 rpm +4°C. The pellet was washed with 70% EtOH, and centrifugation was re-applied for 10 minutes. The ribonucleoprotein complexes were suspended in 0.1 mL of MilliQ water, and samples were stored at -20°C.

The rRNA was purified with phenol-chloroform extraction. The samples were kept on ice, and 1% SDS-containing phenol was added to the samples. Samples were vortexed vigorously for 10 s, kept on ice for 5 min, and centrifuged at 16,200xg at +4°C. The water phase was transferred to a new microfuge tube into which chloroform:phenol mixture (1:1) was added and vortexed for 10 seconds. This step was repeated, using only chloroform to avoid phenol carryover. The water phase was transferred to a new microfuge tube, and the RNA was precipitated with 2.5 vol. ethanol at -20°C for 1 hour. The pellet was washed with 70% EtOH and dried at room temperature for 5 minutes. The purified RNA was dissolved in ultra-pure distilled water.

Total RNA Purification using hot phenol extraction

The strains were grown in LB at 37°C. 10-12 mL of cell culture were transferred to a 15 mL Falcon tube, pelleted via centrifugation at 8000xg for 3-4 minutes, snap-frozen in liquid nitrogen, and stored at -80°C until RNA purification. Total RNA was purified with hot phenol-chloroform extraction, as described previously (Kasari *et al.* , 2013).

Denaturing Agarose Gel Electrophoresis

The isolated RNA samples were separated by denaturing 1.5% agarose gel containing 1xMOPS buffer and 2% formaldehyde. 5 μg of RNA (no 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, #SM1821 and Low Range RNA ladder, # SM1831) were denatured at 55°C for 15 minutes. The RNA mixes were then cooled on ice. Sample loading dye (5μL, 1:6) (0.25% bromophenol blue, 40% sucrose) was added to the samples, and the samples were loaded onto the gel. The electrophoresis buffer was the same as the buffer used to prepare the gel, 1 x MOPS. During the first hour, 60V was applied, and the voltage was increased to 85V.

After 5 hours, when the run ended, the ladder region was cut off and stained for 30 min in the running buffer containing 10000x diluted Diamond Nucleic acid dye (Promega). The transfer of the RNA from the agarose gel to the nylon membrane (Amersham Hyband-N+, GE Healthcare, #RPN303B) was done via capillary

transfer of RNA from the denaturing agarose gel to the nylon membrane (Sambrook, 2001). UV crosslinking was applied to achieve RNA crosslinking to the nylon membrane.

The hybridization of the Northern Blot Membrane

20-25 mL hybridization buffer (0.5 M Sodium phosphate buffer pH 7.2 containing 7% SDS) and the rotating bottle were heated in a hybridization oven (Hybrigene, #Z649570) at 62°C in darkness. The membrane was placed in the bottle and rotated for two hours. The fluorescent-labelled DNA oligonucleotide was added to 10 µM final concentration, and hybridization occurred overnight. The next day, the wash buffer (20 mM sodium phosphate buffer pH 7.2 containing 1% SDS) was warmed in a water bath to 43°C. The membrane was washed with this pre-warmed buffer in a temperature-controlled orbital shaker in a metal box, preventing light exposure. The membrane was washed thrice for 5 minutes with approximately 250 mL of the wash buffer at 43°C. Finally, the membrane was placed into a plastic envelope. The scanning of the membrane was done in the Amersham Typhoon laser scanner.

Statistical analysis

Two-sided Student's t-test with unequal variances was done in GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). Other statistical analyses were done in R vers. 4.2.1, using the Brms package v. 2-18-0 for the Bayesian modelling (Burkner, 2018). For joint multilevel modelling of mean colony radiuses and their standard deviation, the model employing Student's t likelihood was, in brms model language, brm(bf(Radius~Strain*temp + (1|day) + (1|plate), sigma~Strain*temp + (1|day)+ (1|plate)), data=full, family = student(), prior= c(prior(normal(0, 5), class=b), prior(normal(0,2), class="sd"), prior(normal(0,2), class="b", dpar="sigma"))). For mixture modelling of mean colony radiuses, the model description is brm(Radius~Strain*temp + (1|day) + (1|plate), data=full, family = mixture(gaussian, gaussian)). The alamarBlue and corresponding OD_{600} measurements shown in Fig. 1 were modelled with splines using the blmss package version 1.1-8 (Umlauf *et al.* , 2021). The modelling was done separately for each strain and condition (Alamar signal and OD_{600} signal). The model description is bamlss(value ~ s(Time_min), family="gaussian"). The growth curves in Fig. 8C were done using the LOESS smoother in the ggplot2 package v. 3.4.0 geom_smooth function (Wickham, 2016; Wickham *et al.* , 2019).

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AUTHOR CONTRIBUTIONS

U.M., T.T. conceived the study. İ.S., Ü.M., and T.T. designed the research. İ.S., S.R., E.A. and A.Ž. conducted the experiments. İ.S., Ü.M., and M.P. analysed the data. İ.S. prepared the figures and tables. İ.S. and Ü.M. wrote the manuscript. All authors read and approved the manuscript.

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FIGURE AND TABLE LEGENDS

Fig. 1. Growth phenotypes of *[?]ybeX* strain and compensation with single-copy plasmid. (**A**) The *E. coli ybeZYX-lnt* operon chromosomal organization with designed sigma factors (σ^{32} and σ^{24}). (**B**) Dot spot assay with wild type (WT) and *[?]ybeX* strain, along with the wild type strain harbouring empty plasmid (WT/pEmpty), *[?]ybeX* strain transformed with empty plasmid, and *[?]ybeX* strain conjugated with YbeZ, YbeY, or YbeX-expressing single copy TransBac library plasmid. LB agar plates without any antibiotics were incubated at 37°C or 42°C. (**C-D**) The stationary phase outgrowth of wild type and*[?]ybeX* strains harbouring empty plasmid in liquid LB medium. Panel C shows the OD_{600} signal, and panel D shows the alamarBlue fluorescence reading normalized to one. Individual measurements from independent experiments, each presented as the mean value of three technical replicates, are shown as dots. Curves are presented as modelled splines, and 95% credible intervals are shown as shaded areas (see Materials and methods for details). pybeX, pybeZ and pybeY denote the YbeX, YbeZ and YbeY expressing plasmid.

Fig. 2. Characterization and quantification of *[?]ybeX* and Keio wild-type (WT) strain colony sizes at 37degC and 42degC. (**A**) Visual inspection of colony appearance of WT and [?]ybeX strains on LB agar plates. The cells were grown in LB or MOPS minimal media, serially diluted, and plated on LB

agar plates. The plates were incubated at 37degC overnight. (**B-C**) Density plots of the distribution of quantified colony radiuses of *[?]ybeX* and isogenic WT strains at 37degC and 42degC. (**D**) Colony counts for WT and *[?]ybeX* strains grown in LB or MOPS MM are presented. Diluted cells were plated on LB agar plates and incubated overnight at 37degC or 42degC.

Fig. 3. *[?]ybeX* cells exhibit severe sensitivity to sub-lethal concentrations of ribosome-binding antibiotics. (**A**) BW25113 (WT) and *[?]ybeX* cells were grown overnight in LB liquid medium, serially diluted and spotted on LB agar plates supplemented with sub-inhibitory concentrations of indicated antibiotics or without antibiotics (No AB). The plates were incubated at 37degC overnight. (**B**) Representative plates from a dot spot assay with strains described in Fig. 1 are presented. pybeX denotes the YbeX expressing plasmids.

Fig. 4. Growth phenotypes of *[?]ybeX* are growth-phase dependent. (**A**) Experimental scheme for bacterial growth in LB medium. Overnight cultures were directly used for the Stationary phase experiments, while the cells were diluted into fresh LB and regrown for Exponential phase experiments. (**B**) Dot spot experiments of Keio wild-type (WT) and *ybeX* , *ybeZ* , and *ybeY* deletion strains are given. The plates were incubated at 37degC, except for the 42degC plate. (**C** and **D**) Growth curves of indicated *E. coli* strains grown on 96-well plates. The monitored growth of stationary (**C**) and exponential (**D**) phase cells of wild type and *ybeY* , *ybeZ* , and *ybeX* deletion strains in liquid LB medium at 37degC. The growth curves are presented as curves for four biological replicates from two independent experiments, where error bars represent the 95% CI-s.

Fig. 5. Accumulation of ribosomal RNA fragments in stationary phase *[?]ybeX* cells. (**A**) Denaturing agarose gel electrophoresis of hot phenol extracted total RNA samples from wild type and *ybeY* , *ybeZ* , and *ybeX* deletion strains. The empty triangle marks the accumulated shortened rRNA species. (**B**) Northern blot analysis of hot phenol extracted total RNA samples from wild type and *ybeY* , *ybeZ* , and *ybeX* deletion strains. The membrane was hybridized with 16S rRNA targeting oligonucleotide.

Fig. 6. Deletion of *ybeX* leads to the accumulation of distinct rRNA species. (**A**) rRNA operon illustration with locations of the Cyanine 5 (red star) labelled oligonucleotides. (**B**) Sucrose gradient profiles of WT and *[?]ybeX* strains grown at 37degC for 2 hours after the OD600 reached 0.3 (see Fig. 7A). 10-30% sucrose gradients were used for sedimentation. The profiles are representative of four independent experiments. (**C -G**) Northern blot hybridization of the same membrane using different Cyanine 5 (Cy5) labelled oligonucleotides (see Table S3). Truncated ribosomal RNA species are annotated as "trunc.".

Fig. 7. Accumulated distinct ribosomal RNA species are formed in vivo. (**A**) An experimental scheme where stationary phase cells (denoted as STAT) were grown to exponential phase (marked as EXP) followed by chloramphenicol (CAM) treatment (7μg/mL) for 2 hours. (**B**) 10-30% sucrose gradient fractionation of clarified WT and *[?]ybeX* strains lysates. (**C -D**) Northern blot analysis of purified rRNA of sucrose gradient fractions separated on denaturing 1.5% agarose gel. The Northern blot was performed using 16S rRNA-specific oligo. The lower panels present the more prolonged exposure of the distinct accumulated rRNA species for more precise visualization.

Fig. 8. Magnesium supplementation rescues the *[?]ybeX* phenotypes in various growth media. (**A**) The WT and *[?]ybeX* cells were grown overnight in LB, SOB growth media, or LB supplemented with 10 mM $MgCl_2$. The cells were serially diluted and spotted on LB agar plates. The plates with antibiotics were incubated at 37°C. There are also controls without antibiotics, denoted "No AB", at 37°C and at 42°C, as indicated in the first two sub-panels. (**B**) A single colony of WT or *[?]ybeX* was grown overnight in the magnesium-limited peptide-based medium (PBM). 0 μM denotes no $MgCl_2$ supplementation; otherwise, PBM is supplemented with 50, 100 and 200 μM $MgCl_2$. (**C**) WT or *[?]ybeX* cells were grown overnight in a defined MOPS minimal medium supplemented with indicated concentrations of $MgCl_2$ and 0.3% glucose as a carbon source. The outgrowth from these stationary phase cultures was done in MOPS minimal medium supplemented with 525 μM of $MgCl_2$ (this is the prescribed optimal magnesium concentration of the 1x MOPS minimal medium). The regrowth of the cells was monitored at 37°C using a 96-well plate reader. The

growth curves are presented as LOESS curves for six biological replicates from three independent experiments, where shaded areas represent the 95% CI-s for the fitted LOESS curves.

Fig. 9. The growth transition into the stationary phase leads to the *[?]ybeX* phenotype. (A) A scheme of the experimental setup. A single colony was inoculated into MOPS minimal medium supplemented with 10 mM $MgCl_2$ and grown overnight. The next day saturated cultures were washed three times to remove residual magnesium and regrown in 10 μM $MgCl_2$-containing MOPS. Aliquots for plating on LB agar were taken at 2, 3, 4 and 5.5 hours. The LB agar plates either contained or did not contain antibiotics as shown on panels D and E, and they were incubated overnight at 37 °C or 42 °C. (**B**) The growth of the wild-type and the *[?]ybeX* strains in liquid MOPS medium supplemented with 10 μM $MgCl_2$ as monitored at 600 nm. (**C**) The wild-tpe and *[?]ybeX* cells were grown to saturation overnight in MOPS minimal medium containing the indicated amount of $MgCl_2$. The mean optical densities of four biological replicates are shown with 95% CI-s. (**D**) The*[?]ybeX* cells had a growth phenotype only when collected for the outgrowth spot assay at the 5.5h time point. **(E)** When the outgrowth spot assay plates contained tetracycline, erythromycin or chloramphenicol (at subinhibitory concentrations listed in Materials and Methods), the growth phenotype seen at the 5.5h time point was more severe.