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Ribosomal mutations affecting the translation of genes that use non-optimal codons

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Genes that are laterally acquired by a new host species often contain codons that are non-optimal to the tRNA repertoire of the new host, which may lead to insufficient translational levels. Inefficient translation can be overcome by different mechanisms, such as incremental amelioration of the coding sequence, compensatory mutations in the regulatory sequences leading to increased transcription or increase in gene copy number. However, there is also a possibility that ribosomal mutations can improve the expression of such genes. To test this hypothesis, we examined the effects of point mutations in the endogenous ribosomal proteins S12 and S5 in *Escherichia coli*, which are known to be involved in the decoding of the mRNA, on the efficiency of translation of exogenous genes that use non-optimal codons, *in vivo*. We show that an S12 mutant in *E. coli* is able to express exogenous genes, with non-optimal codons, to higher levels than the wild-type, and explore the mechanisms underlying this phenomenon in this mutant. Our results suggest that the transient emergence of mutants that allow efficient expression of exogenous genes with non-optimal codons could also increase the chances of fixation of laterally transferred genes.

Introduction

In all organisms there is a preference towards the use of a specific subset of codons, referred to as 'optimal codons', which usually constitute 70–90% of the codons in a gene and match the most abundant tRNA in the cell [1–3]. Previous studies in *Escherichia coli* [3–7] have shown that optimal codons increase the accuracy of tRNA selection (translation accuracy) and also the total amount of the produced protein (translation efficiency). A possible explanation for these phenomena is that, by using codons that are non-optimal to the tRNA pool, translation accuracy is reduced due to higher rates of amino acid misincorporation and elongation is slowed down due to increased translational proofreading resulting in lower translation efficiency [3–12].

The accuracy of translation does not reach the theoretical maximum and can be modified by antibiotics or mutations in ribosomal proteins that involve the decoding of the mRNA codons into amino acids [13–32]. In *E. coli*, for example, antibiotics such as streptomycin (STR) and spectinomycin (SPEC) were shown to bind to the ribosome and modify the accuracy of translation [13–30]. Moreover, many studies in *E. coli* [13–17,31,32] have shown that alterations in the *rpsL* (the

Abbreviations
ACC, acetyl-CoA carboxylase; Amp, ampicillin; CAI, codon adaptation index; CAT, chloramphenicol acetyltransferase; CHL, chloramphenicol; CHL^R, chloramphenicol resistance; H-NS protein, histone-like nucleoid structuring protein; IPTG, isopropyl thio-β-D-galactoside; LB, lysogeny broth; SPEC, spectinomycin; $SPEC^R$, spectinomycin resistance; STR, streptomycin; STR^R, streptomycin resistance; tAI, tRNA adaptation index; tmRNA, transfer-messenger RNA.

gene encoding the small subunit ribosomal (r) protein S12) produce streptomycin resistance (STR^R) and error-restrictive ribosomes (i.e. ribosomes with reduced translational error rates). In contrast, mutations in the *rpsE* (the gene encoding the small subunit r-protein S5) produce spectinomycin resistance ($SPEC^R$) and error-prone ribosomes (i.e. ribosomes with increased translational error rates).

Two common *rpsL* mutations in *E. coli* that confer STR^R are the replacement of the amino acid lysine 42 with threonine (referred to as K42T) and the replacement of the amino acid lysine 87 with arginine (referred to as K87R) [17,31]. While the K42T mutation also results in error-restrictive ribosomes [18], the K87R mutation results in semi error-restrictive ribosomes, with a translational error rate that is lower than that of wild-type strain yet higher than error-restrictive strains such as the K42T mutant [33]. On the other hand, it was demonstrated that a single amino acid substitution in the *E. coli rpsE* gene of a highly conserved glycine to an aspartate at position 28 (referred to G28D), which produces $SPEC^R$, also resulted in error-prone ribosomes [32].

In this study, we were interested in exploring the possibility that mutations in the decoding centre of the ribosome that are known to affect accuracy will alter the efficiency of translation of genes with non-optimal codons. Towards this aim, we generated three ribosome mutations in *E. coli* K-12 strain MG1655 that differ in their levels of accuracy: the two RpsL mutations K42T (error-restrictive) and K87R (semi error-restrictive), and the RpsE mutation G28D (error-prone). Then we examined the effect of each of these mutations on the level of expression of exogenous genes that encode the same protein but differ in their DNA sequence, compared with wild-type strain. Our results revealed that the semi error-restrictive mutation (K87R), within the *rpsL* gene, caused an increase in the abundance and activity of proteins encoded by exogenous genes containing non-optimal AT-rich codons.

Results

Generation of ribosome mutants in *E. coli* K-12 MG1655 strain

To avoid any potential problems associated with gene disruption and expression while generating the K42T or K87R mutations in the endogenous *rpsL* gene and the G28D mutation in the endogenous *rpsE* gene in *E. coli* K-12 MG1655 strain, we used the one-step allele exchange (Materials and methods). To isolate candidates for K42T or K87R *rpsL* mutations, selection was achieved by plating on medium containing 100 $\mu g \cdot mL^{-1}$ of STR at 37 °C. Similarly, candidates for G28D mutations were selected by plating on medium containing 100 $\mu g \cdot mL^{-1}$ of SPEC. After 2 days, three candidates for a K42T mutation, seven candidates for a K87R mutation and 11 candidates for a G28D mutation were identified and validated by DNA sequencing. To rule out secondary mutational events, these resistance-conferring mutations were then transferred to the wild-type strain using transduction with the phage P1 followed by sequencing of the genomic *rpsL* or *rpsE* genes of the transductants (Materials and methods; see also Table 1). Additionally, transduction with the phage P1 of all three replacements to a wild-type MG1655 strain revealed that the K42T and K87R replacements provided resistance to 100–300 $\mu g \cdot mL^{-1}$ of STR to the wild-type strain, and the G28D replacement provided resistance to 100 $\mu g \cdot mL^{-1}$ of SPEC to the wild-type strain (Table 1).

The influence of the ribosome mutations on the expression of exogenous bacterial genes

To test our hypothesis that ribosome mutations could increase the expression of genes with non-optimal codons, we examined the influence of the *rpsL* (K42T, K87R) and the *rpsE* (G28D) mutations on the expression of orthologous *accA* genes from three evolutionary distant bacteria: the native *E. coli* (class Gammaproteobacteria, referred to as $accA_{Ec}$), *Agrobacterium tumefaciens* (class Alphaproteobacteria, referred to as $accA_{Ag}$) and *Listeria monocytogenes* (class Bacilli, phylum Firmicutes, referred to as $accA_{Lm}$). These genes encode the alpha subunit of carboxyl transferase. Carboxyl transferase is part of a larger complex, acetyl-CoA carboxylase (ACC), which is located in the cytoplasm and catalyses the first and rate-limiting step of fatty acid synthesis [34–36]. Both

Table 1. Position of mutations in S12 and S5 proteins of *E. coli* K-12. The position numbering originates from the start codon of the open reading frame.

Strain	Gene	Position of mutation in *rpsL*/*rpsE* gene	Amino acid replacement	Antibiotic resistance
K42T	*rpsL*	128 (A → C)	Lys to Thr	100–300 $\mu g \cdot mL^{-1}$ STR
K87R	*rpsL*	263 (A → G)	Lys to Arg	100–300 $\mu g \cdot mL^{-1}$ STR
G28D	*rpsE*	84 (G → A)	Gly to Asp	100 $\mu g \cdot mL^{-1}$ SPEC

Table 2. The model genes used in this study and their characteristics. *accAEc, accA* from *E. coli*; *accAAg, accA* from *Agrobacterium tumefaciens*; *accALm accA* from *Listeria monocytogenes*; CAT_{opt}, a CAT variant optimized for expression in *E. coli*; $CAT_{AT\text{-}rich}$, a CAT variant with deoptimized AT-rich codons; $sfGFP_{opt}$, an sfGFP variant optimized for expression in *E. coli*; $sfGFP_{AT\text{-}rich}$, a sfGFP variant with deoptimized AT-rich codons, Based on the average codon usage of a set of highly expressed genes in *E. coli*. Folding energy in the first 40 codons of the gene (Kcal/mole).

Gene	tAI	CAI	Percentage of rare codons	%A + T	Folding energy	Length (bp)	Protein molecular weight (kDa)
$accA_{Ec}$	0.29	0.7	1.25	47.7	−4.7	960	37
$accA_{Ag}$	0.26	0.57	1.57	40.1	−3.1	954	37
$accA_{Lm}$	0.23	0.38	9.71	60.4	−2.6	957	37
CAT_{opt}	0.29	0.97	0.42	49.0	−2.1	708	25
$CAT_{AT\text{-}rich}$	0.2	0.33	9.32	67.4	−1.8	708	25
$_{sf}GFP_{opt}$	0.35	0.95	0	49.7	−5	837	26.9
$_{sf}GFP_{AT\text{-}rich}$	0.26	0.52	7.11	61.3	−5	837	26.9

$AccA_{Ag}$ and $AccA_{Lm}$ protein homologs have 50–52% identity and 69–70% similarity to $AccA_{Ec}$. However, according to our analysis (Materials and methods), while the native $accA_{Ec}$ codon usage is optimized for expression in *E. coli*, the GC-rich $accA_{Ag}$ gene has intermediate codon adaptation values for expression in *E. coli* and the $accA_{Lm}$ uses AT-rich codons that are highly non-optimal compared with *E. coli*, i.e. the gene has low tRNA adaptation index (tAI) and codon adaptation index (CAI) values and a high percentage of rare codons (Table 2). The three *accA* genes were cloned into a pBAD vector containing a tightly regulated arabinose promoter (Materials and methods). A FLAG epitope (DTKDDDDK) was added at the C-terminus [37] and the levels of soluble AccA proteins from all four strains were compared using western blot analysis (Materials and methods).

As shown in Fig. 1A, within all strains the soluble protein levels of $AccA_{Lm}$ were lower than those of $AccA_{Ec}$ and $AccA_{Ag}$. This is in agreement with the low codon adaptation values of $accA_{Lm}$ and despite the fact that it has the weakest predicted mRNA folding energy of the three genes (Table 2), a property that has been shown to be a strong predictor of *E. coli* protein abundance [38]. Thus, the lower protein yield of the non-optimal AT-rich $accA_{Lm}$ gene, in each strain, probably indicates a correlation between codon usage and the level of protein expression [1,3–12,39–44]. However, when comparing the levels of all three proteins between the wild-type strain and the ribosome mutants

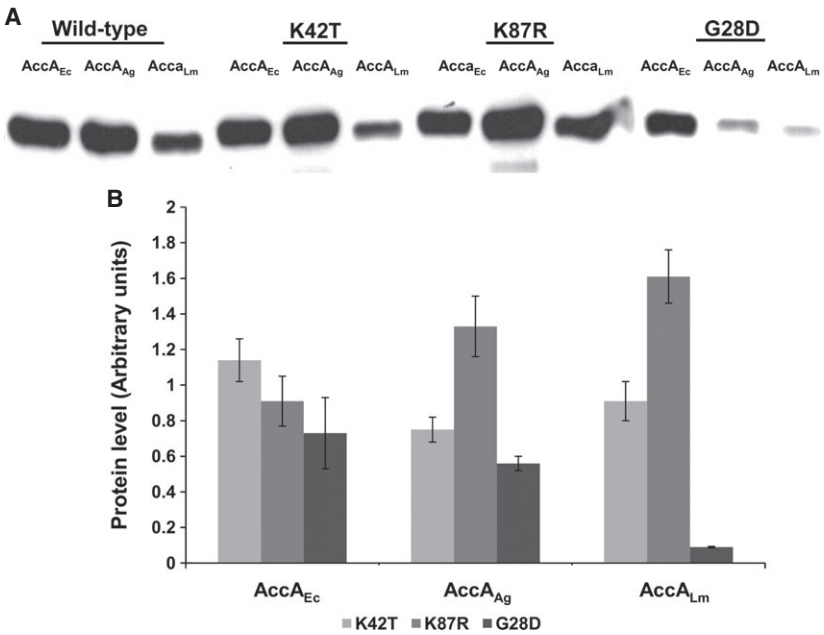

Fig. 1. A comparison of the soluble protein levels of AccA proteins in *E. coli* wild-type and ribosome mutants. (A) Equal quantities of soluble AccA proteins extracts were loaded in all lanes. The levels of expression of the genes in each strain were compared using western blotting with an antibody specific for the FLAG epitope engineered into AccA. (B) Densitometry results are normalized to the values of the wild-type strain and are an average of three independent experiments, one of which is presented in (A). The error bars represent the standard deviation.

(Fig. 1B), although no substantial difference in the levels of soluble $AccA_{Ec}$ was observed, the expression of soluble $AccA_{Ag}$ varied between strains. While the K87R (semi error-restrictive) mutation resulted in an increase of over 1.33 ± 0.17 fold of soluble $AccA_{Ag}$ protein levels, relative to the wild-type, the K42T (error-restrictive) and G28D (error-prone) mutants had soluble $AccA_{Ag}$ protein levels that were lower by 1.33 ± 0.07 and 1.78 ± 0.04 fold, respectively, than the wild-type. Notably, while there was no significant difference in the levels of soluble $AccA_{Lm}$ (which uses AT-rich codons that are highly non-optimal to *E. coli*) between the K42T mutant and the wild-type, the K87R mutant had over 1.61 ± 0.15 fold higher levels of this protein than its parental strain. In contrast, in the G28D mutant there was a reduction of 11 ± 0.004 fold in the expression of soluble $AccA_{Lm}$ compared with the wild-type. These results indicate that the *rpsL* mutation K87R can increase the relative expression levels of $accA_{Ag}$ and $accA_{Lm}$ genes that use non-optimal codons. Since we were interested mainly in increased expression of genes with non-optimal codons, we focused on the K87R mutants in the following experiments.

The K87R mutation reduces protein aggregation of the foreign AccA homologs

Codons requiring rare tRNAs are known to cause translation errors [3], which may result in improper folding and often protein aggregation [10]. Indeed, western blot analysis of non-soluble (aggregated) AccA proteins (Materials and methods) revealed that within both wild-type and K87R strains the amounts of aggregated $AccA_{Ag}$ and $AccA_{Lm}$ proteins were higher than aggregated $AccA_{Ec}$ (Fig. 2). In the wild-type strain the quantities of non-soluble (aggregated) $AccA_{Ag}$ and $AccA_{Lm}$ proteins were 1.52- and 1.65-fold higher than aggregated $AccA_{Ec}$, respectively, while in the K87R strain the amounts of aggregated $AccA_{Ag}$ and $AccA_{Lm}$ proteins were 1.47- and 1.7-fold higher than aggregated $AccA_{Ec}$, respectively. This finding is in agreement with previous studies suggesting that optimal codons might be preferentially used in genes as a result of pressure for translational accuracy [45–50]. It is therefore likely that the K87R mutation, known to confer more accurate translation, would result in more soluble $AccA_{Ag}$ and $AccA_{Lm}$ proteins, due to fewer translational errors and reduced aggregation [10]. Indeed, western blot analysis of aggregated AccA proteins revealed that the amount of aggregated $AccA_{Ag}$ and $AccA_{Lm}$ proteins were 1.44- and 1.53-fold lower in the K87R mutant compared with the wild-type, respectively (Fig. 2). Thus, some of the increase in soluble $AccA_{Ag}$ and $AccA_{Lm}$ proteins in the mutant strain is likely to be due to a lower level of aggregated protein, presumably due to the lower error rate.

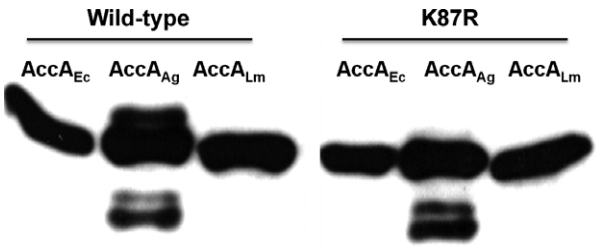

Fig. 2. Comparative western blot analysis of aggregated AccA proteins. Western blotting was performed with equal quantities of total aggregated cellular protein with an antibody specific for the FLAG epitope engineered into AccA. It represents five independent experiments.

The influence of K87R mutation on the expression of synthetic genes that encode the same protein using different codons

Since the analysis of the expression of natural AccA homologs compared the levels of proteins with slightly different amino acid sequences and lengths (Table 2), we followed this assay with a more refined experimental tool, comparing identical proteins encoded by different gene variants. In the additional assays we compared the translation levels of two synonymous versions of the chloramphenicol acetyltransferase (*CAT*) gene (CAT_{opt} and $CAT_{AT\text{-}rich}$) [51] and two synonymous versions of the synthetic 'superfolder' ($_{sf}GFP$) gene ($_{sf}GFP_{opt}$ and $_{sf}GFP_{AT\text{-}rich}$). The $_{sf}GFP$ gene encodes for the synthetic 'superfolder' green fluorescent protein, which has been shown to have a high folding efficiency and a low tendency to aggregate [52]. Hence, $_{sf}$GFP is a preferred protein to study translation efficiency. Where CAT_{opt} and $_{sf}GFP_{opt}$ are optimized for expression in *E. coli* (based on the average codon usage of a set of highly expressed genes in *E. coli* [54]), both $CAT_{AT\text{-}rich}$ and $_{sf}GFP_{AT\text{-}rich}$ variants are enriched with AT-rich codons that were designed to be deoptimized for expression in *E. coli* (Table 2). The engineered *CAT* genes were cloned to a pUC57 plasmid under the control of the *lac* promoter and were immuno-tagged with an AU1 (MDTYRYI) epitope at their N-termini [52]. The $_{sf}GFP$ genes were cloned to the pIA160 plasmid under a P_{trc} promoter and were also immuno-tagged with an AU1 (MDTYRYI) at the C-terminus (Materials and methods).

Comparing the relative expression levels of these *CAT* genes by western blot analysis revealed that the level of soluble $CAT_{AT\text{-}rich}$ protein was lower than that

of the CAT_{opt} protein, in both strains (Fig. 3A). Similarly, the levels of $_{sf}GFP_{opt}$ gene expression were higher than those of the $_{sf}GFP_{AT\text{-}rich}$ gene in both strains (Table 3). Again, differences in mRNA folding energy could not explain these findings, since the $CAT_{AT\text{-}rich}$ gene has less negative mRNA folding energies in the first 40 codons of the gene compared with CAT_{opt} (Table 2). In addition, both $_{sf}$GFP variants have the same mRNA folding energies in their first 40 codons (Table 2). These results indicate again a correlation between usage of more optimal codons and the level of protein expression [1,3–12,39–44].

Importantly, while there was no substantial difference in soluble CAT_{opt} protein levels between the wild-type and the K87R mutant, the level of the soluble $CAT_{AT\text{-}rich}$ protein was 1.88-fold higher in the mutant strain than in the wild-type (Fig. 3B). In addition, comparing the relative expression levels of $_{sf}GFP_{opt}$ by fluorescence (Materials and methods) did not show substantial differences between the wild-type and the K87R mutant (Table 3 and Fig. 3C). However, the levels of $_{sf}GFP_{AT\text{-}rich}$ were about 1.54-fold higher in the K87R mutant than in the wild-type strain (Table 3 and Fig. 3C). These experiments again indicate that the K87R mutation, in *rpsL*, can increase the levels of proteins encoded by genes that use non-optimal codons.

The increased expression of AT-rich gene with non-optimal codons in the *rpsL* K87R mutant results from higher mRNA levels

Notably, unlike $accA_{Lm}$, we did not observe any aggregation of either *CAT* or $_{sf}GFP$ gene products, so improved accuracy by itself cannot explain the differences between the wild-type and the K87R mutant. Therefore, the improvement in protein yields of proteins encoded by genes with non-optimal AT-rich codons, *in vivo*, by the K87R mutation can be caused either directly by improved efficiency of translation or indirectly through effects on mRNA abundance, mediated by the coupling between transcription and translation in bacteria [55]. Quantification of the mRNA levels of all three model genes – $accA_{Lm}$, $_{sf}GFP_{AT\text{-}rich}$ and $CAT_{AT\text{-}rich}$ – using real-time quantitative PCR (Materials and methods) revealed a direct correlation between the level of translation and the corresponding mRNA levels of these genes in the K87R mutant relative to the

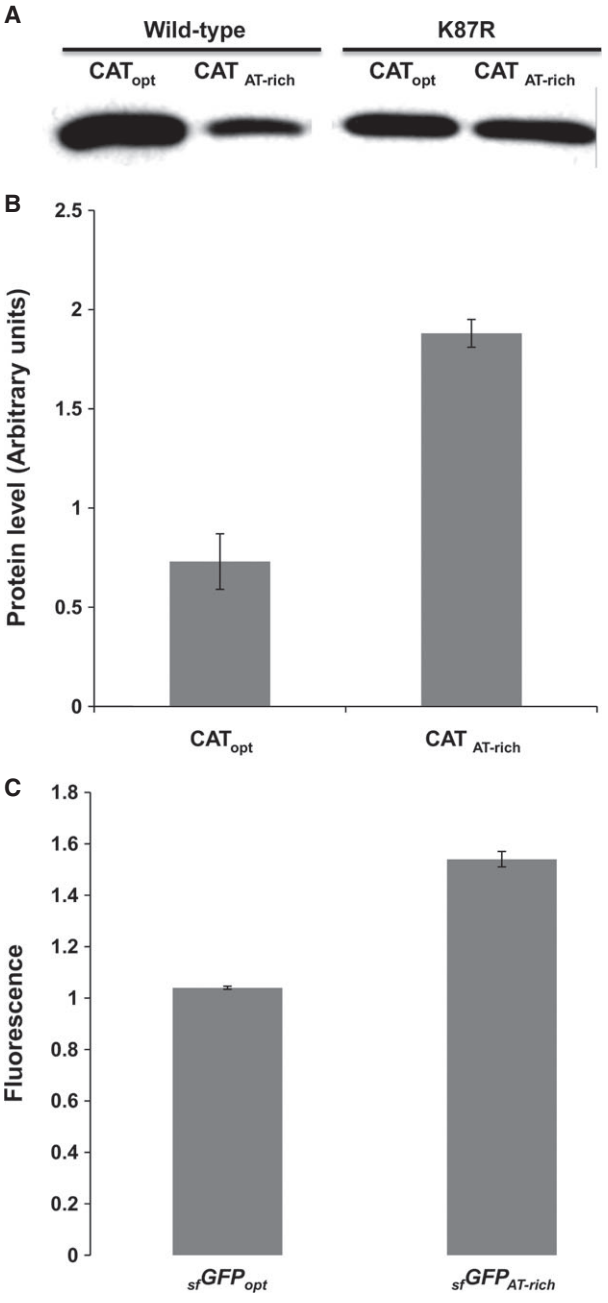

Fig. 3. Comparative analysis of CAT gene expression in *E. coli* wild-type and K87R ribosome mutant using (A) western blotting of equal quantities of total soluble cellular protein extract with an Ig specific for the epitope AU1, as quantified by (B) densitometry and normalized relative to expression in the wild-type strain. Densitometry results are normalized to the values of the wild-type strain and are an average of three independent experiments, one of which is presented in (A). The error bars represent the standard deviations. (C) A comparison of the fluorescence levels of $_{sf}$GFP proteins in *E. coli* wild-type and K87R ribosome mutant. Fluorescence was excited at 488 nm for all $_{sf}$GFP variants. Fluorescence emission was detected at 510 nm (Materials and methods). Results are normalized to the values of the wild-type strain and are an average of three independent experiments. The error bars represent standard deviations.

Table 3. A comparison of the fluorescence levels of $_{sf}$GFP proteins in *E. coli* wild-type and K87R ribosome mutant. The expression levels of $_{sf}GFP_{opt}$ and $_{sf}GFP_{AT\text{-}rich}$ were measured by liquid culture whole-cell fluorescence measurements. Values are mean ± standard deviations. Fluorescence was excited at 488 nm for all $_{sf}$GFP variants. Fluorescence emission was detected at 510 nm (Materials and methods).

		$_{sf}$GFP variant	
Strain	No plasmid	$_{sf}GFP_{opt}$	$_{sf}GFP_{AT\text{-}rich}$
Wild-type	480 ± 32	16414 ± 167	12164.4 ± 590
K87R	334.6 ± 17	17350 ± 814 (1.05)	18733.2 ± 247 (1.54)

wild-type. Importantly, the transcript levels of $accA_{Lm}$, $_{sf}GFP_{AT\text{-}rich}$ and $CAT_{AT\text{-}rich}$ were 1.47, 1.92 and 2.5 times higher respectively in K87R than in its parent strain (Fig. 4). Thus, the differences in mRNA levels can explain most of the observed differences between the K87R strain and the wild-type in the levels of proteins encoded by genes with non-optimal codons.

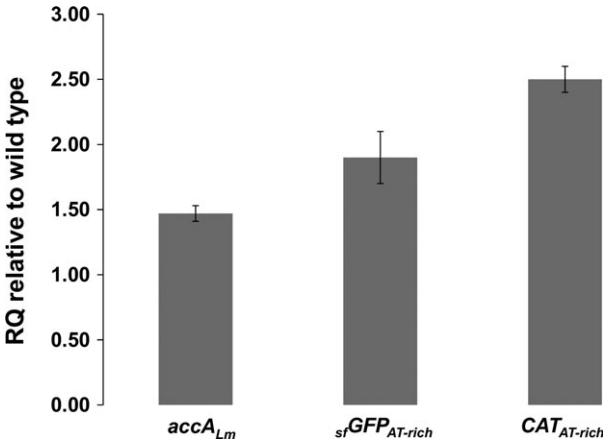

Fig. 4. The mRNA levels of $accA_{Lm}$, $_{sf}GFP_{AT\text{-}rich}$ and $CAT_{AT\text{-}rich}$ genes in the K87R strain relative to the mRNA level of this gene in the wild-type. The mRNA level was quantified using real-time quantitative PCR (Materials and methods). Results are normalized to the values of the wild-type and are an average of three independent experiments. The error bars represent the standard deviations.

Increased antibiotic resistance of the K87R mutant expressing the deoptimized $CAT_{AT\text{-}rich}$ gene

In the light of our findings, we were interested to test the effect of the increased $CAT_{AT\text{-}rich}$ protein and mRNA levels in the K87R mutant on the specific activity of $CAT_{AT\text{-}rich}$ protein, using the FAST CAT® (deoxy) assay (Materials and methods). We found that while there was no difference in the specific activity of CAT_{opt} between K87R and the wild-type strains, the $CAT_{AT\text{-}rich}$ specific activity was 1.4 times higher in K87R compared with the wild-type (Fig. 5).

The increased $CAT_{AT\text{-}rich}$ protein activity in the mutant could also manifest itself in increased resistance to chloramphenicol (CHL^R), which is mediated by the enzyme CAT. To experimentally test this hypothesis, we characterized the growth properties of both strains in rich liquid medium (LB) at 37 °C (Materials and methods). As expected [18–23,55,56], the *rpsL* mutant (without any plasmid) grew slightly slower than the wild-type in the absence of antibiotics. As shown in Table 4, the doubling times of the wild-type and K87R were 30.5 ± 3 and 35 ± 3 min, respectively. Growth experiments in LB medium supplemented with increasing amounts of CHL revealed that 2 μg·mL^{-1} of CHL was the lowest concentration that completely inhibited the growth of both strains. However, when expressing either one of the *CAT* gene variants, both strains showed resistance even at 10 μg·mL^{-1} of CHL (Table 4). Predictably (and in agreement with [57]), there was a direct correlation between the codon usage of the gene and the doubling time within the wild-type: under all tested conditions, the growth rate of the wild-type expressing the $CAT_{AT\text{-}rich}$ was longer than the wild-type expressing the CAT_{opt} gene (Table 4). Surprisingly, in the K87R strain an opposite trend was

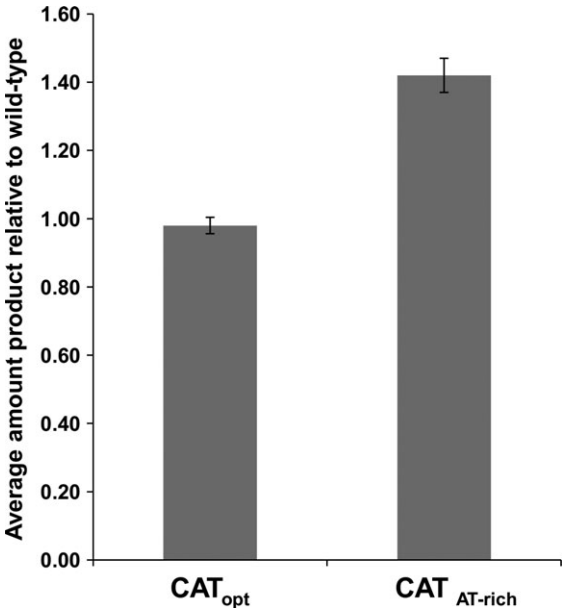

Fig. 5. Enzymatic activity of the soluble CAT protein in the wild-type and K87R strains, measured by FAST CAT® at 37 °C. Results are normalized to the values of the wild-type and are an average of three independent experiments. The error bars represent the standard deviations.

Table 4. The generation time (in minutes) of wild-type and *rpsL* mutants in LB medium supplemented with different concentrations of CHL. Numbers indicates the mean of generation time (minutes) ± standard deviation of three independent experiments. NG, no growth.

		CHL concentration ($\mu g \cdot mL^{-1}$)						
		0	1	2	4	6	8	10
No plasmid	Wild-type	30.5 ± 3	37 ± 1			NG		
	K87R	35 ± 3	52 ± 1					
CAT_{opt}	Wild-type	35 ± 1	43 ± 1	44 ± 1	50 ± 1	51 ± 1.5	50 ± 1.2	61 ± 7
	K87R	37 ± 3	43 ± 1	52 ± 3	58 ± 8	64 ± 10	71 ± 15	74 ± 4
$CAT_{AT\text{-}rich}$	Wild-type	36 ± 5	44 ± 3	46 ± 1	59 ± 1	61 ± 4	73 ± 16	81 ± 18
	K87R	37 ± 0	39 ± 3	40 ± 3	44 ± 0	54 ± 4	64 ± 11	68 ± 8

observed: under selection of CHL, the doubling time of the K87R mutant carrying the $CAT_{AT\text{-}rich}$ gene was slightly shorter than the same strain carrying the CAT_{opt} gene. Moreover, when the CAT_{opt} variant was expressed, in the presence of increasing concentrations of CHL, the wild-type had faster growth rates (shorter generation times) than K87R. However, K87R expressing $CAT_{AT\text{-}rich}$ gene showed a significantly higher growth rate than the wild-type only under CHL selection (Table 4). These findings raise the possibility that the ribosomal mutation conferring antibiotic resistance, such as the K87R mutation, which emerges during STR exposure, could also result in a higher expression of other antibiotic resistance genes using non-optimal codons, thus providing an increased fitness phenotype for those mutants.

Discussion

Proper function of proteins is crucial for the viability of all organisms. Therefore, organisms invest a considerable amount of energy to carry out efficient and accurate translation of proteins [58–67]. However, genetic and biochemical evidence indicates that the accuracy of translation by the ribosome is not at its maximal level, but is rather at its optimal level for the rate of bacterial growth [68–70]. In this study we tested whether ribosome mutations in proteins of the decoding centre, which are known to alter the translational accuracy of the ribosome, could also increase translation of exogenous genes with non-optimal codons *in vivo*.

Primarily, our results revealed that a ribosome mutation in *E. coli*, namely a K87R substitution in *rpsL*, can increase expression of three different exogenous genes encoded by non-optimal codons, by 1.7-fold on average. Given that codons requiring rare tRNAs are known to cause translational errors [49], it is possible that the increased expression of genes with non-optimal codons by the semi error-restrictive mutant K87R is related to the accuracy of translation by the ribosome. When translating exogenous genes with deoptimized AT-rich codons, this mutant makes fewer translational errors, which result in fewer error-ridden misfolded proteins, which might eventually be degraded [8–12]. The finding that the error-prone mutation (in *rpsE*) resulted in a decrease in translation of the AT-rich $accA_{Lm}$ supports this assumption. Additionally, $AccA_{Lm}$ exhibited a reduced level of aggregation in the K87R mutant, although the *CAT* and $_{sf}GFP$ genes did not show any sign of aggregation in any strain tested, presumably because they are highly soluble proteins. All the proteins we tested were comparatively short, and larger multi-domain proteins that rely on co-translational folding may be much more sensitive to errors in translation and thus more affected by modifications to the accuracy of the ribosome. While laterally acquired genes are generally shorter than the genomic average [71,72], the exceptions, such as multi-domain enzymes, are very interesting proteins and should be the focus of future study.

Interestingly, increased expression of the foreign AccA orthologous was not displayed by the K42T mutant, which is known to have higher fidelity than K87R [17]. Since both mutations, K42T and K87R, are in the same r-protein, S12, a possible explanation for the different translational phenotype of genes with AT-rich non-optimal codons between them can be that these S12 mutations result in dissimilar structural alterations to the ribosome that differently affect the translation of genes with AT-rich non-optimal codons. Notably, an *in vitro* study in *E. coli* [17] has shown that increased expression of exogenous genes with non-optimal codons was observed in a K42T but not in a K87R mutant in a cell-free transcription and translation system. Accordingly, it might be suggested that there are some cellular factors, which affect the translation of genes with non-optimal codons *in vivo* but not *in vitro*, that operate differently in the K87R mutant, resulting in higher transcript levels.

Further investigation of the mechanism leading to increased expression of genes with non-optimal codons by the K87R mutant revealed that this improvement

could be mostly attributed to a variation in transcript levels. Quantifications of the mRNA levels revealed that, on average, AT-rich non-optimal mRNA levels were nearly 2-fold higher in the K87R strain than in the wild-type strain. Several mechanisms can explain the increased levels of mRNA of genes with non-optimal codons by the K87R *rpsL* mutant. One mechanism could be decreased 'xenogeneic silencing' by the histone-like nucleoid structuring (H-NS) protein in the K87R mutant. Recent studies in *E. coli* [54,73–77] have shown that H-NS can selectively recognize and silence the transcription of exogenous AT-rich DNA, in a process known as xenogeneic silencing. Since in bacteria there is a coupling between transcription and translation [54], it is possible that the K87R mutation, which changes the rate of translation [13–17], also changes the rate of transcription. Hence the length of the gap between the ribosome and the RNA-polymerase is affected and results in inhibition of the binding of H-NS to the AT-rich mRNA. Consequently there are more AT-rich mRNA molecules in K87R and hence the increased translation in K87R compared with the wild-type. A second mechanism that can explain the increased mRNA and protein levels of the AT-rich genes in the K87R mutant is related to transfer-messenger RNA (tmRNA) activity [78]. Bacteria use tmRNA activity to release stalled ribosomes, allowing incomplete polypeptides to be tagged and rapidly degraded by ATP-dependent proteases. Ribosome pausing can be caused by an absence of a stop codon but also by cognate tRNA shortage, due to use of rare codons. Thus, ribosome stalling at non-optimal codons can activate the tmRNA system [79–81] and result in mRNA cleavage. Since $accA_{Lm}$, $_{sf}GFP_{AT\text{-}rich}$ and $CAT_{AT\text{-}rich}$ genes contain a multitude of non-optimal and rare codons, the chance for mRNA cleavage within the ribosome's A-site and subsequent tmRNA activity is high specifically for those genes. Importantly, a recent study revealed that *rpsL* mutants in *E. coli* exhibited reduced A-site mRNA cleavage and tmRNA-SmpB-mediated SsrA peptide tagging compared with wild-type cells [78]. Thus, a reasonable explanation for our observation of increased levels of mRNA of genes with non-optimal codons by the K87R *rpsL* mutant may be a decrease in tmRNA activity compared with the wild-type, which would also explain why genes that utilize common/optimal codons were not differentially expressed.

Translational errors might disrupt protein folding [8–12]. It is possible that the $CAT_{AT\text{-}rich}$ specific activity was higher in K87R compared with the wild-type since fewer translational errors were made by the error-restrictive ribosome than the wild-type ribosome which would result in more properly folded and functional $CAT_{AT\text{-}rich}$ proteins. However, since no aggregated CAT proteins, drop-off events or read-through events were detected, our findings imply that in the K87R mutant higher activity levels of $CAT_{AT\text{-}rich}$ are probably due to higher protein levels rather than higher specific activity per protein molecule.

In view of our findings, we wondered whether such K87R ribosomes existed in nature. In the growth experiments we performed, in agreement with previous studies [17,19–23,55,56], we also observed that the K87R mutation in the *rpsL* gene impaired the growth of bacteria compared with the wild-type. Moreover, *rpsL* mutations that confer semi error-restrictive phenotype were also shown to cause impaired peptide chain elongation and alterations in the efficiency and specificity of protein chain initiation [20,22,23,55]. These findings suggest that there are some negative factors that are more pertinent than the benefit that would arise from increased accuracy, otherwise these mutations would be fixed in many bacterial species. Accordingly, it can be suggested that the accuracy of translation and the speed of translation evolved to be in the right balance required for optimal growth. In conclusion, our findings reinforce the view that the intermediate accuracy of the ribosome is a compromise between a higher efficiency of error-free protein expression and the cost of fitness in maintaining higher levels of accuracy [82].

Lateral gene transfer is a major source of genetic innovation in microbial evolution allowing bacteria to acquire novel functions that can improve their fitness in a given environment. Recently acquired genes typically use a different subset of codons than that of the recipient genome [82], generally using AT-rich codons [83], which could impede their translation. Since the usefulness of a gene relies on its translation into active protein, codon usage compatibility between exogenous genes and recipient genomes may increase the fixation probability of lateral gene transfer events [84,85]. In cases where the exogenous genes are incompatible with the codon usage of the host, inefficient translation can be overcome by different mechanisms, such as gradual amelioration of the coding sequence [86]. However, evidence from experimental evolution approaches has not shown this codon-by-codon optimization, but rather found compensatory mutations in the regulatory sequences leading to increased transcription [51], or increase in gene copy number [87], which have a quicker effect on expression. Nevertheless, this solution is highly risky to the bacterium in question, as high expression of a gene with non-optimal codons has been shown to result in substantial growth inhibition [57]. Here we observed an additional and novel molecular mechanism

that improves the translation of non-optimal genes. We have demonstrated that some ribosome mutations resulted in an increase in expression of AT-rich genes which contain non-optimal codons, and that this can increase antibiotic resistance conferred by these genes.

One may speculate that under stressful conditions, especially when cells are exposed to antibiotics that affect translation, such as STR, resistant mutants with altered ribosomal functionality will spontaneously arise and will express some exogenous genes better than the wild-type bacteria. When these genes are highly beneficial (conferring additional resistance, for example), the chances of fixation of resistance genes will be higher in these mutants. Thus, mutant ribosomes that arise by a single point mutation and are maintained by antibiotic pressure can increase the chances of acquiring additional resistance determinants and other exogenous genes, such as virulence genes. This mechanism can augment the effects of transcription-enhancing mutations that promote expression of laterally acquired genes [51].

Materials and methods

Strains and plasmids used in this work

The bacterial strains and plasmids used in this study are listed in Tables 5 and 6 respectively.

Growth medium

Lysogeny broth (LB) medium was composed of 1% tryptone, 0.5% yeast extract, 0.5% NaCl and solidified by addition of 1.5% agar.

Growth and maintenance of bacterial strains

Generally, overnight liquid cultures were prepared by inoculation of a single colony into LB medium and incubation at 37 °C in a gyratory shaker (New Brunswick Scientific, Edison, NJ, USA), model G-25 at 270 r.p.m. For long-term storage, overnight cultures were mixed with 25% (v/v) glycerol and stored at −70 °C.

Generation of ribosome mutants in *E. coli* K-12 MG1655

The *rpsL* (K42T, K87R) and *rpsE* (G28D) mutations (Table 1) were introduced into the *rpsL* and *rpsE* genes of *E. coli* K-12 MG1655 strain by the one-step allele exchange method [90] using the double-stranded DNAs listed in Table 7. In order to generate the K42T mutation, a point mutation AAA to ACA was introduced into the *rpsL* gene using the double-stranded DNA oligonucleotides RpsL_K42T_sense and RpsL_K42T_antisense (Table 7). To generate the K87R mutation, an AAA to AGA point mutation was introduced into the *rpsL* gene, using the double-stranded DNA oligonucleotides RpsL_K87R_sense and RpsL_K87R_antisense (Table 7). Candidates for *rpsL* mutations were selected using LB 1.5% agar plates supplemented with 100 $\mu g \cdot mL^{-1}$ of STR. The G28D mutation was generated by inserting the point mutation GGT to GAT in the 28th codon of the *rpsE* gene by using the double-stranded DNA oligonucleotides RpsE_G28D_sense and RpsE_G28D_antisense (Table 7). Candidates for *rpsE* mutations were selected using LB 1.5% agar plates supplemented with 100 $\mu g \cdot mL^{-1}$ of SPEC.

To verify that these mutations are the only source of resistance, they were transferred by transduction with the phage P1 to a wild-type MG1655 strain (as described below) and the respective region was amplified and sequenced using the primers RpsL_F and RpsL_R or RpsE_F and RpsE_R (Table 7).

P1 phage transduction

An overnight culture of *E. coli* K-12 MG1655 carrying the genetic modification (donor strain) was diluted 1 : 100 in 2.5 mL of LB supplemented with 5 mM $CaCl_2$. After 1 h of growth with aeration at 37 °C (D_{595} of 0.1–0.2), 100 μL of P1 phage lysate was added to the culture. Growth continued at 37 °C for another 1–3 h until the culture was completely lysed. Three microliters of chloroform were added to the lysate and vortexed to eliminate any intact donor bacteria. The cell debris was centrifuged away at 13000 ***g*** for 2 min and the supernatant was kept at 4 °C. In order to transfer the mutation to recipient strains, an overnight culture of *E. coli* K-12 MG1655 was harvested by centrifugation (2500 ***g*** for 2 min) and resuspended in the original culture volume in fresh LB with 100 mM $MgSO_4$ and 5 mM $CaCl_2$. Several 100 μL aliquots containing serial dilutions of the P1 lysate were incubated with 100 μL of recipient cells for 30 min at 37 °C without aeration and then for 1 h at 37 °C with aeration. Selection for putative transductants was performed with 100 $\mu g \cdot mL^{-1}$ of STR, selecting for K42T or K87R mutants, or with 100 $\mu g \cdot mL^{-1}$ of SPEC, selecting for the G28D mutant.

DNA purification

Genomic DNA was purified by using Promega's Wizard genomic DNA purification kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol.

Polymerase chain reaction (PCR)

All PCR products were amplified using Phusion DNA Polymerase (Finnzymes Oy, Espoo, Finland) with bacterial genomic DNA serving as a template. Reactions were cycled

Table 5. Strains used in this study

Escherichia coli strains	Characteristics	Source or reference
Escherichia coli K-12 MG1655	Wild-type strain	Laboratory collection
K42T	*E. coli* K-12 MG1655 with a point mutation in the amino acid 42, codon 128 within the *rpsL* gene. This point mutation is A to C in second position, from Lys to Thr. Error-restrictive, STR^R	[104]
K87R	*E. coli* K-12 MG1655 with a point mutation in the amino acid 28, codon 261 within the *rpsL* gene. This point mutation is A to G in second position, from Lys to Arg. Semi error-restrictive, STR^R	
G28D	*E. coli* K-12 MG1655 with a point mutation in the amino acid 87, codon 84 within the *rpsE* gene. This point mutation is G to A in second position, from Gly to Asp. Error-prone, $SPEC^R$	This study
Wild-type$^{accA_{Ec}}$	*E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA* gene from *E. coli*, ampicillin resistance (Amp^R)	[37]
Wild-type$^{accA_{Ag}}$	*E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA* gene from *A. tumefaciens*, Amp^R	
Wild-type$^{accA_{Lm}}$	*E. coli* K-12 MG1655 carrying the pBAD24 vector containing an a*ccA* gene from *L. monocytogenes*, Amp^R	
K42T$^{accA_{Ec}}$	K42T *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA E. coli* gene, Error-restrictive, STR^R, Amp^R	This study
K42T$^{accA_{Ag}}$	K42T *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *AccA A. tumefaciens* gene, Error-restrictive, STR^R, Amp^R	
K42T$^{accA_{Lm}}$	K42T *E. coli* K-12 MG1655 carrying the pBAD24 vector containing a *accA L.monocytogenes* gene, Error-restrictive, STR^R, Amp^R	
K87R$^{accA_{Ec}}$	K87R *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA E. coli* gene, Semi error-restrictive, STR^R, Amp^R	
K87R$^{accA_{Ag}}$	K87R *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA A. tumefaciens* gene, Error-restrictive, STR^R, Amp^R	
K87R$^{accA_{Lm}}$	K87R *E. coli* K-12 MG1655 carrying the pBAD24 vector containing a *accA L.monocytogenes* gene, Error-restrictive, STR^R, Amp^R	
G28D$^{accA_{Ec}}$	K87R *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA E. coli* gene, Error-prone, $SPEC^R$, Amp^R	
G28D$^{accA_{Ag}}$	K87R *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA A. tumefaciens* gene, Error-restrictive, STR^R, Amp^R	
G28D$^{accA_{Lm}}$	G28D *E. coli* K-12 MG1655 carrying the pBAD24 vector containing an *accA L. monocytogenes* gene, Error-restrictive, STR^R, Amp^R	
Wild-type$_{sf}^{GFP_{opt}}$	Wild-type *E. coli* K-12 MG1655 carrying the pIA vector containing a $_{sf}GFP_{opt}$ gene, Amp^R	
Wild-type$_{sf}^{GFP_{AT-rich}}$	Wild-type *E. coli* K-12 MG1655 carrying the pIA vector containing a $_{sf}GFP_{AT\text{-}rich}$, gene, Amp^R	
K87R$_{sf}^{GFP_{opt}}$	K87R *E. coli* K-12 MG1655 carrying the pIA vector containing an $_{sf}GFP$ *opt*. Semi error-restrictive, STR^R, Amp^R	
K87R$_{sf}^{GFP_{AT-rich}}$	K87R *E. coli* K-12 MG1655 carrying the pIA vector containing an $_{sf}GFP_{AT\text{-}rich}$. Semi error-restrictive, STR^R, Amp^R	[52]
Wild-type$^{CAT_{opt}}$	Wild-type *E. coli* K-12 MG1655 carrying the pUC57 vector containing a CAT_{opt} gene. Amp^R, CHL^R	
Wild-type$^{CAT_{AT-rich}}$	Wild-type *E. coli* K-12 MG1655 carrying the pUC57 vector containing a CAT AT-rich gene. Amp^R, CHL^R	This study
K87R$^{CAT_{opt}}$	K87R *E. coli* K-12 MG1655 carrying the pUC57 vector containing a *CATopt*. Semi error-restrictive, STR^R, Amp^R, CHL^R	
K87R$^{CAT_{AT-rich}}$	K87R *E. coli* K-12 MG1655 carrying the pUC57 vector containing a $CAT_{AT\text{-}rich}$ gene. Semi error-restrictive, STR^R, Amp^R, CHL^R	

31 times (denaturation at 98 °C, 10 s; annealing at varying temperatures, 30 s; elongation at 72 °C, 30 s). Primers are listed in Table 7.

PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and were sequenced with both primers (Table 7) to locate the genetic alterations associated with STR^R mutations at the *rpsL* gene or with $SPEC^R$ mutations at the *rpsE* gene.

Determination of parameters for the level of optimality of codons of the model genes

Recent studies in *E. coli* have called into question the link between efficient codon usage and protein levels [4,89,90]. We therefore validated our assumption by testing whether proteins encoded by endogenous genes using codons that are non-optimal to the *E. coli* genome (as

Table 6. Plasmids used in this study.

Plasmid	Characteristics	Source
pBAD24	Cloning expression vector, under arabinose promoter araC; origin of replication, pBR322/ColE1, M13; markers, Amp^R; other features, optimized SD sequence	Laboratory collection
pUC57	Cloning expression vector, under the control of the lac promoter; markers, Amp^R	[51]
pIA160	Cloning expression vector, under the control of the of IPTG inducible P_{trc} promoter; markers, Amp^R	Irina Artsimovitch Research Laboratory, Department of Microbiology, Ohio State University, Columbus, OH, USA

Table 7. Oligonucleotides and primers used in this work.

Name	Sequence (5′ → 3′)	Use
RpsL_K42T_sense	GTACTCGTGTATATACTACCACTCCTACAAAACCGAACTCCGCGCTGCGTAAAGTA TGCCGTGTTCGTCT	Allele exchange [85]
RpsL_K42T_antisense	AGACGAACACGGCATACTTTACGCAGCGCGGAGTTCGGTTTTTTAGGAGTGGTAG TATATACACGAGTAC	
RpsL_K87R_sense	CTCCGTGATCCTGATCCGTGGCGGTCGTGTTAGAGACCTCCCGGGTGTTCGTTA CCACACCGTACGTGGT	
RpsL_K87R_antisense	ACCACGTACGGTGTGGTAACGAACACCCGGGAGGTCTCTAACACGACCGCCACG GATCAGGATCACGGAG	
RpsE_G28D_sense	GGTAAACCGCGTATCTAAAACCGTTAAAGGTGATCGTATTTTCTCCTTCACAGC TCTGACTGTAGTTGG	
RpsE_G28D_antisense	GCCAACTACAGTCAGAGCTGTGAAGGAGAAAA TACGAGCACCTTTAACGGTTTT AGATACGCGGTTTACC	
RpsL_Forward	ATGGCAACAGTTAACCAGCT	PCR and sequencing
RpsL_Reverse	CCTTAGGACGCTTCACGC	
RpsE_Forward	ATGGCTCACATCGAAAAACA	
RpsE_Reverse	TTCCCCAGAATTTCTTCAAC	
pUC57_Forward	GTAAAACGACGGCCAGTC	
pUC57_Reverse	GGAAACAGCTATGACCATG	
pBAD_Forward	ATGCCATAGCATTTTTATCC	
pBAD_Reverse	GATTTAATCTGTATCAGG	
k1	CAGTCATAGCCGAATAGCCT	
k	CGGTGCCCTGAATGAACTGC	
GFP_Forward	CACGAACAGACCGAAACCTT	
GFP_Reverse	GGAAGTACGCCAGGTTTT	
$accA_{Ec}$_Forward	ATCTCGGTGCATGGCAGA	RT-PCR
$accA_{Ec}$_Reverse	AAATGCCAGGCGAACGTA	
$AccA_{Ag}$_Forward	GGAGATCAACCGCCTGGA	
$AccA_{Ag}$_Reverse	GCGTTTTCTGCCAAGCAT	
$AccA_{Lm}$_Forward	CCATCCGGAGAGACCTAC	
$AccA_{Lm}$_Reverse	ATGTACGATCCCCGTGAA	
CAT_{opt}_Forward	CACGAACAGACCGAAACCTTC	
CAT_{opt}_Reverse	CGGGAAGTACGCCAGGTTTT	
$CAT_{AT\text{-}rich}$_Forward	TCTTATGCCACTAGGAATACA	
$CAT_{AT\text{-}rich}$_Reverse	TCTTTGGTGTCAGCTCCTCCT	
$_{sf}$GFP_Forward	CACGAACAGACCGAAACCTT	
$_{sf}$GFP_Reverse	GGAAGTACGCCAGGTTTT	
Bla_Forward	CGGTCGCCGCATACACTATT	
Bla_Reverse	CATGCCATCCGTAAGATGCT	
OmpA_Forward	GGCTGCTCTGGATCAGCTGTA	
OmpA_Reverse	TCGGACAGACCCTGGTTGTAA	

opposed to totally exogenous genes) are indeed below the level of detection when their respective genes are transcribed. For that purpose, we obtained data from three independent proteome analyses, performed by Gevaert *et al.* [91], Corbin *et al.* [92] and Taoka *et al.* [93], and divided all coding genes of *E. coli* K-12 into two groups: genes that were transcribed and translated (referred to as detectable) and genes that were transcribed but no protein could be detected (referred to as non-detectable). In order to focus on high-confidence data, only genes whose products were either present in all three studies or absent in all of them were analysed. For each group of genes we then calculated the average values of the tAI, the CAI [94] and the deoptimized codon percentage. The tAI takes into account both tRNA content and codon–anticodon interactions, making it a useful measure for translational accuracy [95,96]. The tAI value is gene-specific and ranges from 0 to 1, where a high tAI value of a particular gene indicates a high number of optimal codons in this gene [95,96]. In our analysis, the tAI was calculated as described in [97] by using the equation described in [96]. The CAI measures the deviation of a given protein coding gene sequence with respect to a reference set of genes, which is composed of highly expressed genes [98,99]. Hence, the CAI provides an indication of gene expression levels under the assumption that there is translational selection to optimize gene sequences according to their expression levels [98,99]. Like tAI, the CAI value is gene-specific and ranges from 0 to 1. A high CAI value of a particular gene indicates a high number of optimal codons in the gene [98,99]. The CAI has been calculated according to the codon usage of *E. coli* K-12 and based on known highly expressed genes of *E. coli* K-12, as defined by Puigbó *et al.* [100]. Examination of codon usage in all 4290 *E. coli* genes reveals a number of codons that are underrepresented [101], in particular the codons for arginine, AGA, AGG and CGA; the codon for isoleucine, AUA; and the codon for leucine, CUA [101]. All these codons represent < 8% of the corresponding population of codons for that amino acid [101]. The codon usage of highly expressed genes demonstrates a more extreme bias in which arginine codons AGA, AGG, CGG and CGA, isoleucine codon AUA, leucine codon CUA, glycine codon GGA and proline codon CCC fall to < 2% of their respective populations [101]. Therefore, an additional and novel way to compare the codon usages of genes is by examining the distribution of non-optimal codons within them.

As shown in Table 8, proteins that were not detectable despite transcription of their respective genes had significantly lower tAI and CAI values and a higher non-optimal codon fraction than genes that produced detectable levels of protein ($P < 0.001$, Mann–Whitney test for each of the three comparisons). Hence, for the purpose of this study we defined a gene as having non-optimal codons for expression in *E. coli* if it met the following criteria: tAI ≤ 0.26, CAI ≤ 0.55, percentage non-optimal codons $\geq 4\%$, with respect to the *E. coli* genome.

Transformation of plasmids

All plasmids carrying the model genes (Table 6) were transformed into wild-type and ribosome mutant strains by standard electroporation and were selected on LB agar plates supplemented with 50 $\mu g \cdot mL^{-1}$ of ampicillin (Amp) at 37 °C. Following selection, the sequences of the model genes were confirmed by sequencing using the primers listed in Table 7.

Extraction of soluble proteins

For extraction of soluble proteins for western blot analysis, cultures of bacteria carrying the plasmids encoding the model genes were grown overnight in LB containing 50 $\mu g \cdot mL^{-1}$ Amp and 0.2% glucose at 37 °C. On the next day all cells were diluted 1 : 100 and were grown in LB containing 20 $\mu g \cdot mL^{-1}$ Amp and 0.2% glucose at 37 °C. When the culture reached an D_{595} of 0.4, 1% of arabinose or 1 mM isopropyl thio-β-D-galactoside (IPTG) was added to the medium in order to induce expression of the genes. After the cells reached an D_{595} of 1 (mid log phase), the total soluble proteins were extracted using BugBuster protein extraction reagent (Novagene, Merck Millipore, USA). Three milliliters of culture were pelleted and resuspended in 150 μL of Bug Buster protein extraction reagent containing 1.4 mM phenylmethanesulfonyl fluoride. Cells were vortexed for 20 min at room temperature and then centrifuged for 10 min at 14 000 ***g*** at 4 °C. The supernatant containing the total soluble protein fraction was analysed by a Bradford assay.

Extraction of protein aggregates

Wild-type or ribosome mutant cells were grown as described above. The extraction of aggregated proteins was performed in two steps. First, in order to extract the total fraction, 1 mL of cells was centrifuged for 3 min at 20 000 ***g*** at room temperature. Then, cells were resuspended in 1 mL of Tris EDTA/phenylmethylsulfonyl fluoride solution. Sonication was performed to lyse the cells,

Table 8. Average of tAI, CAI and rare codons percentage. Numbers indicate the mean ± standard deviation.

Parameter	Detectable	Non-detectable
tAI	0.26 ± 0.02	0.23 ± 0.02
CAI	0.55 ± 0.1	0.44 ± 0.07
Rare codons percentage	2% ± 1.7%	4.18% ± 2.34%

and cells were then centrifuged for 30 min at 20 000 ***g*** at 4 °C. Subsequently, the pellet containing total protein extraction was resuspended in 8 M urea, vortexed for 20 min and centrifuged for 30 min at 20 000 ***g*** at 4 °C to precipitate non-soluble membrane proteins. The supernatant, containing aggregated proteins, was transferred to a fresh micro-centrifuge tube and analysed by Bradford assay [102]. Equal amounts of aggregated proteins were analysed by western blotting and densitometry (EZQuant, Tel-Aviv, Israel).

Protein gels

10% polyacrylamide gels containing 0.2% SDS were used to separate protein samples at a constant voltage of 180 V per gel.

Western blot analysis

To analyse the differences of the soluble proteins between the wild-type and the ribosome mutants, western blot analysis was performed as previously described [38]. Briefly, following SDS/PAGE of cell extracts, the proteins were transferred onto nitrocellulose membranes using a Mini Protean Tetra system (Bio-Rad); the membranes were blocked with 5% (wt/vol) non-fat milk in $NaCl/P_i$ and incubated for 1 h at room temperature. Then, for detection of proteins, primary antibodies were diluted in the blocking buffer and incubated with blots overnight at 4 °C. The nitrocellulose membranes were washed three times for 20 min each with $NaCl/P_i$ containing 0.05% Tween-20 before the addition of a secondary antibody conjugate in blocking buffer for 3 h.

For detection of AccA proteins, we used mouse monoclonal anti-FLAG (R) M2 (dilution 1 : 1000, catalog number F1804 5MG; Sigma, Rehovoth, Israel) as a primary antibody and a horseradish peroxidase conjugated goat anti-mouse as a secondary antibody (dilution 1 : 5000, catalog number 115-035-003; Anko, Israel). For detection of *CAT* and *$_{sf}$GFP* genes we used a rabbit polyclonal antibody anti-AU1 (dilution 1 : 1000, catalog number AB-ab3401; Abcom, Israel) against the N-terminus of the CAT protein [52] and a horseradish peroxidase conjugated goat anti-rabbit IgG (dilution 1 : 5000, catalog number 111-035-003; Jackson, Israel) as a secondary antibody. Detection was performed with EZ-ECL chemiluminescence detection kit (Biological Industries, Israel).

Predictions of mRNA secondary structure minimum free energy

The first 40 nucleotides in each open reading frame were performed by UNAFold: software for nucleic acid folding and hybridization (RNA3 and RNA2.3) [103].

Chloramphenicol acetyltransferase activity assay

CAT assay was performed by using the FAST CAT® Assay Kit F-2900 (Molecular Probes) that utilizes a fluorescent CHL substrate to measure CAT activity. The assay was performed at 37 °C according to the manufacturer with the following modification. To facilitate comparisons, we used the same soluble protein extraction protocol as that described above for western blots.

Growth experiments

To determine the generation times of all strains in the absence of plasmids, cultures that were grown in LB at 37 °C for 10–14 h were diluted 1 : 100 in LB and grown at 37 °C to an D_{595} of 0.8. Subsequently, cultures were diluted to an D_{595} of 0.05. All growth measurements were performed in a 96-well plate, with 200 μL medium per well, incubated for 24 h at 37 °C, with automatic shaking, in a thermostatted plate reader (ELX808 IU-PC– Biotek, BioTek Instruments Inc., USA), and absorbance was monitored at 595 nm (D_{595}) every 20 min. Every growth experiment was performed at least three times.

Liquid culture whole-cell fluorescence measurements

To compare the expression level of different *$_{sf}$GFP* genes, individual cultures of bacteria carrying the pIA plasmids (Table 6) encoding a specific *$_{sf}$GFP* were grown in 3 mL LB medium containing 50 $\mu g \cdot mL^{-1}$ Amp and 0.2% glucose at 37 °C for 10−14 h. After 12 h, all cells were diluted 1 : 100 and grown in 3 mL LB containing 20 $\mu g \cdot mL^{-1}$ Amp and 0.2% glucose at 37 °C. When cultures reached an D_{595} of 0.4, 1 mM of IPTG was added to the medium to induce the expression of the *$_{sf}$GFP* until an D_{595} of 1. Subsequently, cultures were diluted to an D_{595} of 0.05 in 200 μL LB containing 20 $\mu g \cdot mL^{-1}$ Amp and 1 mM IPTG.

All measurements were performed in a thermostatted plate reader (synergy HT-Biotek, BioTek Instruments Inc., Winooski, USA), in a 96-well plate with 200 μL medium per well, with automatic shaking, at 37 °C for 8 h. Growth was monitored by measuring absorbance at 595 nm every 20 min. Fluorescence was measured (488 nm excitation, 510 nm emission, 10 nm band pass for $_{sf}$GFP). In addition we compared the fluorescence ratio to the D_{595}. Every experiment was performed in at least three replicates.

Quantitative real-time PCR analysis

Transcription levels of all tested genes were analysed using real-time quantitative PCR. RNA was harvested from cells grown using the same conditions as used for soluble protein extraction using the RNEasy kit (Qiagen), according

to the manufacturer's protocol. In all cases 2 μg of RNA was reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit® (Applied Biosystems, Jerusalem, Israel), which uses random oligomers. Real-time PCR was performed on 10 ng of cDNA using SYBER Green®, in a Step-one Plus real time PCR system (Applied Biosystems). As reference genes we used both *bla* and *omp* genes. The *bla* mRNA was encoded by pUC57 and the *omp* gene is a chromosomal gene. Statistical analysis was performed using the STEPONE™ V2.1 software. All primers are listed in Table 7.

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Author contributions

Conceived and designed the experiments: GP, IB and UG. Performed the experiments: GP and NS. Analysed the data: GP, AD, TT and UG. Contributed reagents/materials/analysis tools: IGB and TT. Wrote the manuscript: GP and UG.

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