Correcting direct effects of ethanol on translation and transcription machinery confers ethanol tolerance in bacteria


The molecular mechanisms of ethanol toxicity and tolerance in bacteria, although important for biotechnology and bioenergy applications, remain incompletely understood. Genetic studies have identified potential cellular targets for ethanol and have revealed multiple mechanisms of tolerance, but it remains difficult to separate the direct and indirect effects of ethanol. We used adaptive evolution to generate spontaneous ethanol-tolerant strains of Escherichia coli, and then characterized mechanisms of toxicity and resistance using genome-scale DNAseq, RNAseq, and ribosome profiling coupled with specific assays of ribosome and RNA polymerase function. Evolved alleles of metJ, rho, and rpsQ recapitulated most of the observed ethanol tolerance, implicating translation and transcription as key processes affected by ethanol. Ethanol induced miscoding errors during protein synthesis, from which the evolved rpsQ allele protected cells by increasing ribosome accuracy. Ribosome profiling and RNAseq analyses established that ethanol negatively affects transcriptional and translational processivity. Ethanol-stressed cells exhibited ribosomal stalling at internal AUG codons, which may be ameliorated by the adaptive inactivation of the MetJ repressor of methionine biosynthesis genes. Ethanol also caused aberrant intragenic transcription initiation in mRNAs with low ribosome density, which was reduced in a strain with the adaptive rho mutation. Furthermore, ethanol inhibited transcript elongation by RNA polymerase in vitro. We propose that ethanol-induced inhibition and uncoupling of mRNA and protein synthesis through direct effects on ribosomes and RNA polymerase conformations are major contributors to ethanol toxicity in E. coli, and that adaptive mutations in metJ, rho, and rpsQ help protect these central dogma processes in the presence of ethanol.

Aliphatic alcohols such as ethanol are important microbial bioproducts whose toxic effects are known to limit their production in both bacteria and yeast (1–4). Thus, elucidating the mechanisms by which these alcohols exert toxic effects and understanding modes of microbial tolerance are important both to understand basic microbial physiology and to engineer microbes with more efficient fermentative capacities (5, 6). Escherichia coli is a model of choice for these studies. Its well-defined physiology and powerful genetic tools have allowed the identification of multiple effects of ethanol on biomolecules and cellular processes. One well-established toxic effect of ethanol on E. coli is an increase in cell-envelope permeability (7–9). E. coli exposed to ethanol exhibit reduced peptidoglycan cross-linking, which is detrimental to viability, and altered membrane-lipid composition, which may represent an attempt to cope with ethanol stress (10, 11). Ethanol induces broad transcriptional changes in E. coli that extend beyond membrane-stress responses (12, 13), however, suggesting that membrane effects explain only a part of the toxicity of ethanol. Consistent with this idea, widely varied approaches have successfully been used to achieve modest ethanol tolerance in E. coli, including random transposon insertion (14, 15), overexpression of native gene libraries (16–18), overexpression of protein chaperones (19), engineered oxidation of ethanol (20), transcriptional rewiring through the catabolite activator protein (21) or the transcription factor σE (22), and modulation of cellular fatty-acid composition (23). The mechanisms by which these genetic changes confer tolerance remain unclear. Additional mechanistic studies are needed to determine the most physiologically relevant impacts of ethanol and to distinguish primary effects from downstream consequences.

An intriguing possibility raised by previous results is that the fundamental processes of transcription and translation may be affected by ethanol. For instance, biochemical studies have shown that purified E. coli ribosomes are prone to misreading errors when treated with ethanol (24–26) although in vivo effects of ethanol on translational error have not been reported. Also, ethanol-treated E. coli produce the alarmone (pp)pGpp (27), a signal of translation inhibition (28). Detrimental effects of

Significance

Microbiologically produced aliphatic alcohols are important bio-commodities but exert toxic effects on cells. Understanding the mechanisms by which these alcohols inhibit microbial growth and generate resistant microbes will provide insight into microbial physiology and improve prospects for microbial bio-technology and biofuel production. We find that Escherichia coli ribosomes and RNA polymerase are mechanistically affected by ethanol, identifying the ribosome decoding center as a likely target of ethanol-mediated conformational disruption and showing that ethanol inhibits transcript elongation via direct effects on RNA polymerase. Our findings provide conceptual frameworks for the study of ethanol toxicity in microbes and for the engineering of ethanol tolerance that may be extensible to other microbes and to other short-chain alcohols.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE56408). Lists of noncoding RNA regions, pseudogenes, and gene sets used in ribosome profiling analysis are available from GEO under accession no. GSE56372.

*Present address: Department of Microbiology and Immunology, University of California, San Francisco, CA 94143.

1To whom correspondence should be addressed. E-mail: landick@biochem.wisc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1401853111/-/DCSupplemental.

1073/pnas.1401853111

www.pnas.org/cgi/doi/10.1073/pnas.1401853111

Published online June 9, 2014

E2576–E2585 | PNAS | Published online June 9, 2014

www.pnas.org/cgi/doi/10.1073/pnas.1401853111

PNAS
ethanol on transcription are suggested by two independent reports linking decreased Rho activity to ethanol tolerance in *E. coli* by a point mutation in rho (14, 15) or by inhibition of Rho activity by overexpression of YaeO/Repo (18). Rho is a hexameric RNA helicase that can terminate transcription when it is not coupled with translation (29). Reduced Rho activity leads to numerous changes in gene expression that might confer ethanol tolerance (14, 30). The reported effects of ethanol on ribosomes and (p)ppGpp induction suggest that uncoupling of translation from transcription could additionally cause maladaptively high Rho termination within genes in ethanol-stressed cells.

We sought to identify the primary effects of ethanol on *E. coli* and to understand mechanisms of tolerance using a two-stage strategy. First, we selected for tolerance-conferring mutations by directed evolution, allowing cultures to accumulate spontaneous mutations while minimizing bias against mutations in essential genes that can accompany transposon mutagenesis or overexpression. Second, we tested for physiological effects of ethanol suggested by the evolved alleles and investigated the mechanisms by which evolved alleles counteracted ethanol toxicity. Our results demonstrate that ethanol has detrimental effects on central dogma processes in vivo, including increases in translational error, ribosome stalling, and intragenic Rho-dependent transcription termination, as well as on transcription elongation in vitro. Mutant alleles isolated in this study help to ameliorate in vivo effects of ethanol, underscoring the physiological relevance of transcription and translation in ethanol toxicity and resistance.

**Results**

Mutations in *metJ*, *rho*, and *rpsQ* Confer Ethanol Tolerance and Improve Fermentative Yields. To reveal physiologically important targets of ethanol, we performed serial-passage evolution experiments to select for spontaneous mutations that conferred a growth advantage to *E. coli* in minimal glucose medium containing increasing concentrations of ethanol up to 65 g/L (Fig. 1A). We isolated clonal strains from the evolved cultures by growing culture aliquots on nonselective plates and selecting single colonies for further study. We tested ethanol tolerance of clonal strains by following growth in media containing 40–65 g of EtOH/L and selected the three highly tolerant isolates MTA156, MTA157, and MTA160 for genomic sequencing (Table 1). MTA156 and MTA160 were independently isolated from a single evolved culture whereas MTA157 was isolated from a separate culture evolved in parallel. Mutations present in these strains (Table S1) affected genes involved in various pathways, but six of the eleven mutant alleles encoded variant proteins with clear ties to transcription and translation, including two variants of transcription termination factor Rho, two variants of the master repressor of methionine synthesis (MetJ), a variant ribosomal protein S17 (RpsQ), and a variant of the conserved transcription elongation/termination factor NusA.

We selected for detailed study a single clonal strain, MTA156, which displayed improved growth in the presence of ethanol (Fig. 1B and Fig. S1) comparable with that reported for highly ethanol-tolerant *E. coli* strains described by various groups (1, 17, 18, 20, 22). Genomic resequencing of MTA156 identified mutations affecting *ispB*, *iptF*, *metJ*, *rho*, *rpsQ*, and *topA* (Table 1 and Table S1). Replacement of these loci with wild-type alleles reduced ethanol tolerance in each case except that of *topA* (Fig. S1A).

Tolerance conferred by mutations in genes involved in LPS transport (iptF) and quinone biosynthesis (*ispB*) likely act by ameliorating effects of ethanol on processes occurring in the *E. coli* cell envelope. The other three tolerance-conferring alleles had clear ties to transcription and translation: *metJ*Δ*E91*, *rho*Δ*L270M*, and *rpsQ*Δ*H31P* (Table 2). Combining these evolved alleles in an otherwise wild-type background improved aerobic

**Table 1. Strains used in the current study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td><em>E. coli</em> K-12 F- Δ<em>ivg</em> rfb-50 rrfl-1</td>
<td>(83)</td>
</tr>
<tr>
<td>EP23</td>
<td>MG1655 <em>metJ</em>Δ[ΔE91]</td>
<td>This study</td>
</tr>
<tr>
<td>EP49</td>
<td>MG1655 <em>rpsQ</em>Δ[ΔH31P]</td>
<td>This study</td>
</tr>
<tr>
<td>EP61</td>
<td>MG1655 <em>metJ</em>Δ[ΔE91] <em>rho</em>Δ[ΔL270M] <em>rpsQ</em>Δ[ΔH31P]</td>
<td>This study</td>
</tr>
<tr>
<td>MTA376</td>
<td>MG1655 Δ<em>ldhA</em>::FRT pJGG2 (<em>Z. mobilis</em> pdc adhB)</td>
<td>This study</td>
</tr>
<tr>
<td>MTA722</td>
<td>MTA376 <em>metJ</em>Δ[ΔE91] <em>rho</em>Δ[ΔL270M] <em>rpsQ</em>Δ[ΔH31P]</td>
<td>This study</td>
</tr>
<tr>
<td>RL2325</td>
<td>MG1655 <em>rho</em>Δ[ΔL270M]</td>
<td>This study</td>
</tr>
<tr>
<td>RL2739</td>
<td>MG1655 <em>rpsQ</em>Δ[ΔL150]</td>
<td>This study</td>
</tr>
</tbody>
</table>

Fig. 1. Selection of mutations conferring ethanol tolerance. (A) Summary of directed evolution experiment, with number of serial passages at each step of increasing ethanol concentration indicated. (B) Representative growth curves showing response of MG1655, MTA156, and ethanol-tolerant triple mutant EP61 to ethanol addition. Mean growth rates for pre- and post-stress cultures are shown ± SEM for four biological replicates. Time points noted were used for sampling in ribosome-profiling experiments. Wild-type MG1655 and the ethanol-tolerant triple mutant EP61 were sampled before ethanol addition (T0), during acute stress (T1), and during chronic stress (T2).
growth in the presence of ethanol to a level near that of MTA156 (Fig. 1B and Fig. S1A). The triple mutant (strain EP61) also showed increased ethanol tolerance under anaerobic conditions (Fig. S1 C and D). An ethanologenic strain bearing the evolved metJ, rho, and rpsQ alleles produced 30% more ethanol than the base strain on a per-cell basis in a synthetic mimic of lignocellulose hydrolysate, indicating that the ethanol-tolerant phenotype can promote biofuel production under industrially relevant conditions (Fig. S1E).

The tolerance associated with mutations in metJ, rho, and rpsQ implicated translation and transcription as targets of ethanol. MetJ represses expression of genes involved in methionine biosynthesis (31). Rho terminates transcription uncoupled from translation, halting mRNA synthesis when translation terminates prematurely (29). RpsQ is ribosomal protein S17, an essential component of the 30S subunit that plays a role in maintaining translational accuracy (32). We focused on phenotypes related to these three targets to study the effects of ethanol on transcription and translation within the cell.

**Ethanol Induces Toxic Translational Misreading.** The evolved rpsQ [H31P] allele is identical to the nea301 rpsQ allele previously selected as a neamine-resistance mutation (33). The nea301 allele encodes a variant of protein S17 that increases translational accuracy in vitro in the presence of ethanol and other misreading agents (25). The in vivo effects of ethanol on translational accuracy have not been reported, but isolation of a hyperaccurate ribosomal mutation led us to hypothesize that ethanol would stimulate translational misreading, which would in turn be reduced by the evolved rpsQ allele.

We tested this hypothesis by measuring misreading in the presence of varying concentrations of ethanol using a sensitive assay developed by Kramer and Farabaugh (34). In this assay, an active-site codon of firefly luciferase (F-luc) is mutated (K529N) such that it produces an inactive enzyme if correctly translated; if ribosomal error leads to insertion of a lysine at this position, an active enzyme is produced instead. The firefly luciferase is translationally fused to a wild-type jellyfish luciferase active under different chemical conditions, which was used to control for changes in transcription/translation rate, protein stability, and other factors. All measurements of mutant F-luc activity were normalized to an isogenic strain expressing wild-type F-luc fused to jellyfish luciferase to control for effects of ethanol on the two enzymes. Ethanol caused a dose-dependent increase in normalized F-luc activity expressed by wild-type E. coli, up to ninefold higher than untreated controls at 40 g of EtOH/L (Fig. 2A), representing an increase in the miscoding error rate similar to that previously reported for inhibitory concentrations of streptomycin (34). Strikingly, the ethanol-tolerant phenotype triple mutant and an otherwise wild-type strain bearing the rpsQ[H31P] allele showed dramatically reduced levels of F-luc activity at all concentrations of ethanol, such that F-luc activity in the mutant strains grown at 40 g of EtOH/L was similar to wild-type cultures without ethanol. These results demonstrate that ethanol increases translational misreading in vivo and that the evolved rpsQ allele strongly reduces translational misreading in the presence or absence of ethanol.

We assessed the physiological importance of ethanol-induced errors in protein synthesis by measuring synergistic toxicity between ethanol and antibiotics that affect different steps in translation. Unrelated stressors at sublethal concentrations generally have multiplicative effects on culture growth when combined whereas stressors that are functionally related vary from this pattern (35, 36). When combined with streptomycin, an antibiotic that induces translational misreading, ethanol caused approximately fourfold greater toxicity than predicted by the multiplicative model for unrelated stressors (Fig. 2B). In contrast, ethanol did not show cooperative synergy with chloramphenicol, which inhibits peptidyl transfer in the ribosome but does not decrease translational accuracy (37), suggesting that ethanol does not

### Table 2. Selected mutations conferring ethanol tolerance

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Measured effect of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>metJ</td>
<td>AAAGAGATC→AAGATC</td>
<td>ΔE91</td>
<td>Up-regulation of methionine biosynthesis gene expression (Table S2)</td>
</tr>
<tr>
<td>rho</td>
<td>CTG→ATG</td>
<td>L270M</td>
<td>Reduced transcription termination by Rho (Fig. S3)</td>
</tr>
<tr>
<td>rpsQ</td>
<td>CAC→CCC</td>
<td>H31P</td>
<td>Increased accuracy during protein synthesis (Fig. 2A)</td>
</tr>
</tbody>
</table>
impede the peptidyl transfer reaction. These results strongly indicate that sublethal ethanol stress induces physiologically damaging levels of translational misreading, perhaps compromising the conformation of the ribosomal decoding site known to be targeted by streptomycin (38, 39).

The observed synergy between ethanol and streptomycin suggested that ribosomal proteins other than RpsQ classically implicated in translational accuracy, like RpsL (protein S12), might also play a role in ethanol tolerance. Consistent with this hypothesis, we observed that the rpsL150 allele (40), which confers streptomycin resistance, increased fitness during growth at a modest concentration (20 g/L) of ethanol (Fig. S2). Ethanol has also been reported to rescue streptomycin-dependent mutants with lesions mapping at or near rpsL (41), which generally display a hyper-accurate translation phenotype (42), implying a potent effect of ethanol on ribosome decoding activity.

Ribosome Profiling Reveals Ethanol Induction of Ribosomal Termination. Translational misreading has been shown to cause ribosome stalling and termination (43), and (p)ppGpp production in ethanol-accelerated translation (44), which generally display a hyper-accurate translation phenotype (42), implying a potent effect of ethanol on ribosome decoding activity.

![Diagram of RNAseq and ribosome footprinting](image)

Fig. 3. Ribosome profiling reveals ethanol-induced ribosome halting and Rho activity. (Top) A summary of ribosome profiling sample treatment for side-by-side RNAseq and ribosome footprinting, adapted from Ingolia et al. (44). (A–C) Relative mRNA signal levels from 5’ to 3’ for groups of ORFs: “all genes” (the 3,048 genes represented in mRNA and ribosome footprint datasets), high ribosome density quintile (610 genes), and low ribosome density quintile (610 genes). (D–F) Relative ribosome occupancy signal levels (ribosome footprint signal divided by mRNA signal) from 5’ to 3’ for groups of ORFs as in A–C. Data from wild-type (MG1655) cultures are shown in gray squares, and data from the ethanol-tolerant triple mutant (EP61) cultures are shown in colored triangles. Error bars show SEM. T0, T1, and T2 represent prestress, acute stress, and chronic stress conditions (Fig. 1B).

We designed the ribosome-profiling experiment to compare three physiological conditions apparent in ethanol-stress experiments (Fig. 1B): logarithmic growth before ethanol stress (T0), the period of growth cessation due to acute stress (T1), and the phase in which cells had resumed growth under chronic ethanol stress (T2). Practical and cost limitations on the number of samples processed precluded examining the single and double rho, metI, and rpsQ mutants individually; thus, we limited the experiment to comparing the triple mutant to wild type at the three indicated time points.

To assess ribosome processivity, we examined the change in relative ribosome occupancy from the 5’ end to the 3’ end of ORFs, dividing each ORF into eight even segments and averaging signals across sets of genes. Decreases in relative occupancy at the 3’ end of messages would indicate aberrant translational termination between the 5’ and the 3’ end of the ORF. Because ribosome occupancy is the ratio of site-specific ribosome abundance over site-specific mRNA abundance, we first quantified mRNA signals from RNAseq across ORFs (Fig. 3 A–C) and then used the ratio of raw ribosome footprint signal to mRNA signal to generate plots of ribosome occupancy (Fig. 3 D–F). To facilitate genome-wide comparisons, we identified a set of 3,048 protein-coding genes with unambiguously mapped reads in all RNAseq and ribosome footprinting datasets. Initially, we looked for evidence of translation termination by averaging relative occupancy values on various mRNAs and ribosome abundance along the lengths of mRNA transcripts.

![Diagram of Ribosome Profiling](image)

![Diagram of mRNA and Ribosome Density](image)
across this set of “all genes” (Fig. 3D). Ethanol-stimulated translational termination was evident and followed similar patterns in both wild-type and mutant cultures. Before stress, ribosomes were widely distributed across mRNAs. During acute stress (T1), relative ribosomal occupancy near the 3’ ends of genes dropped from ~0.95 to ~0.75. After cells resumed growth under chronic stress (T2), genome-wide ribosome occupancy shifted back to near prestress patterns. These observations indicate that the acute stress phase, which is coupled to growth cessation, was characterized by widespread aberrant termination of translation within mRNAs. By the time growth resumed, both strains had largely corrected this defect.

In analyzing the ribosome profiling data, we observed that mRNAs with higher prestress ribosome density exhibited greater decreases in 3’ ribosome occupancy. To assess this pattern further, we separated the “all gene” set into quintiles based on ribosome density in unstressed wild-type cells and examined the upper and lower quintiles. The upper ribosome density quintile exhibited a decrease in occupancy at the 3’ end of genes that was, on average, greater in magnitude than the analogous decrease in the set of all genes (Fig. 3E). This result indicates that a high density of ribosomes on the message could not prevent ethanol-induced ribosomal halting. The low ribosome density quintile, in contrast, did not exhibit such a decrease in ribosome occupancy (Fig. 3F). Although this flatter ribosome occupancy curve could indicate a smaller effect of ethanol on translation, low ribosome density mRNAs, a likely alternative explanation is that translation termination on these messages might be coupled to termination of transcription. Because ribosome occupancy is ribosome abundance corrected for mRNA abundance, parallel decreases in both would appear as relatively flat ribosome occupancy across a message, as observed for the low-density quintile.

Coupling of ethanol-induced translation termination to transcription termination could explain why we and others found that mutations reducing Rho activity confer ethanol tolerance. Rho terminates transcription of untranslated mRNA molecules; thus, poorly translated mRNAs might be particularly susceptible to ethanol-induced Rho-dependent termination because an ethanol-induced increase in ribosomal termination could not be compensated by other ribosomes on the mRNA. We therefore used the RNAseq data to assess changes in intragenic transcription termination after ethanol treatment.

**Rho-Dependent Transcription Termination Is Increased During Ethanol Stress.** Intragenic transcription termination results in decreased mRNA levels from the 5’ end to the 3’ end of ORFs. We tested for this pattern by dividing the RNAseq reads into the same gene sets used to analyze ribosome occupancy (Fig. 3A–C). In the “all gene” gene set, we observed statistically significant decreases in 3’ abundance relative to 5’ abundance, indicative of transcriptional termination, during acute stress for both wild type and the mutant (T1) (Fig. 3A). This change was markedly greater in magnitude for wild-type cells, suggesting that the variant Rho expressed by the mutant may reduce intragenic transcription termination. However, mRNAs with high ribosome density did not exhibit obvious ethanol-induced decreases in 3’-proximal signal (Fig. 3B), suggesting that high ribosome density may protect transcripts from termination.

The low ribosome-density gene set exhibited a similar pattern to the set of all genes, but with stronger and more persistent effects (Fig. 3C). For these genes, mRNA levels were maximal at the 5’ end and dropped as transcription proceeded toward the 3’ end in both strains. The decrease in mRNA level after the 5’ end of the gene was greatest during acute ethanol stress and only partially recovered during chronic stress, indicating that ethanol inhibited the ability of cells to produce full-length transcripts of these genes even after cells had resumed active growth. Although both strains exhibited this general pattern of transcription termination, the tolerant mutant had a clear advantage in producing full-length transcripts, maintaining significantly higher mRNA levels across the length of genes than wild type at all time points.

These results suggested that Rho[L270M] reduced transcription termination. To test this possibility, we performed an RNAseq analysis of a strain containing only the rho[L270M] mutation. The experiment confirmed the reduced rho activity phenotype, revealing increased readthrough of a set of previously defined Rho-dependent terminators (45) in the rho[L270M] strain compared with wild type (Fig. S3). Of 114 Rho-dependent terminators for which fold change could be reliably calculated using our RNAseq dataset (Dataset S1), 84% exhibited increased terminator readthrough in the mutant, and nearly half (45%) exhibited a twofold or greater increase (Mann–Whitney U test; P < 0.001). Thus, the rho[L270M] mutation confers a global defect in Rho-dependent transcription termination.

We concluded that, during ethanol stress, Rho aberrantly terminates transcription of hundreds of genes. RNA polymerases (RNAPs) synthesizing mRNAs with fewer ribosomes than average were more prone to Rho-dependent termination. Especially for these poorly translated mRNAs, the reduced activity of Rho [L270M] favored production of full-length transcripts. Thus, our results are consistent with the hypothesis that ethanol-induced translational termination uncouples translation from transcription, stimulating Rho to terminate mRNA elongation.

**Ethanol Inhibits mRNA Synthesis by RNA Polymerase in Vitro.** Another possible way ethanol could increase Rho-dependent transcription termination is to slow transcript elongation by RNA polymerase, which would increase the time available for Rho action. To investigate this possibility, we tested whether modest concentrations of ethanol could affect transcript elongation by RNAP in vitro in the absence of translation. In the presence of ethanol at concentrations of 30 g/L or 60 g/L, the average rate of transcript elongation by *E. coli* RNAP was reduced by 10–30%, and transcriptional pausing at a subset of sites was exacerbated (Fig. 4). Thus, ethanol not only may increase chances for Rho loading by causing translational misreading and termination, but also may increase Rho action by slowing RNAP and thus increasing the kinetic window within which Rho can effect termination.

**Ethanol Inhibits Translation of Nonstart AUG Codons.** The results described above (see *Ribosome Profiling Reveals Ethanol Induction of Ribosomal Termination*) strongly implicated ribosome stalling in the toxic effect of ethanol on *E. coli*. Such stalling could be random or could be biased toward specific sites in the genome. We hypothesized that ribosomes in ethanol-treated cells might be prone to stop at AUG codons due to methionine limitation because a mutation in the master repressor of methionine biosynthesis, *metI*, contributed to ethanol tolerance. We therefore used the ribosome profiling data to assess ethanol-induced changes in ribosome occupancy at start and nonstart AUG codons with codons for other amino acids and stop codons (Fig. 5). Increased codon occupancy in the ribosome profiles reflects increased dwell time of ribosomes at those codons relative to others, thus revealing codons whose translation was inhibited by ethanol.

Ethanol had small effects on ribosome occupancy at most codons, but strongly affected occupancy at nonstart AUG codons. Nonstart AUG occupancy dramatically increased during acute toxicity (T1) and only partially recovered during chronic toxicity (T2) in both strains. This pattern correlates with the overall pattern of translation termination observed by ribosome profiling (Fig. 3D), which was strongly induced at T1 but largely recovered by T2. The magnitude of the ethanol effect on nonstart AUG occupancy was less for the mutant than for wild type at both time points (Wilcoxon matched-pairs rank test; P < 0.001). We infer that the *metI*Δ*E91* allele protects against ethanol stress at least in part because it leads to increased expression of methionine biosynthesis genes (Table S2), potentially increasing the methio-
nine pool available to the cell. Consistent with this hypothesis, deletion of the metJ repressor or addition of excess methionine improved ethanol tolerance of wild-type E. coli (Fig. S4).

In contrast to nonstart AUG codons, ribosome occupancy at AUG start codons increased in the mutant after ethanol addition but decreased in the wild-type strain (Fig. 5). In principle, relative start codon occupancy reflects the rate of translation initiation relative to elongation (i.e., how much time that ribosomes spend at start codons relative to other codons). Because rates of translation initiation and growth are positively correlated in E. coli (46), these different responses likely reflect the lesser effect of ethanol on mutant versus wild-type growth rates (<3× versus >6×, respectively) (Fig. 1B).

**Discussion**

Our study of the effects of ethanol on central dogma processes was motivated by the discovery that significant ethanol tolerance in E. coli could be recapitulated by mutations in genes encoding translation initiation and growth.

---

**Fig. 4.** Ethanol slows transcription in vitro. (A) Representative gel lanes showing in vitro transcript elongation in the presence of 0 g, 30 g, or 60 g ethanol/L. Elongation complexes (10 nM) were halted at the end of a 26-nt C-less cassette and then exposed to ethanol. NTPs (30 μM each) were added subsequently, and products from 2-min, 4-min, and 8-min extensions were resolved by gel electrophoresis. RO indicates template run-off products. Dots to the left of the gel panels indicate the mean transcript length of RNA products for the experiment shown, and vertical lines show regions containing 15% of the signal upstream and downstream from the mean. (B) Densitometric traces of gel lanes in A. Height of traces corresponds to relative densitometric signal at a given nucleotide position. Mean average transcript lengths from two independent experiments are shown.

**Fig. 5.** Ethanol-induced changes in ribosome occupancy by encoded amino acid. Log2 values of mean ethanol-induced ribosome occupancy changes for codons encoding the indicated amino acid are shown. Changes were measured as the ratio of poststress occupancy to prestress occupancy. Data from wild-type (MG1655) cultures are shown in blue, and data from the ethanol-tolerant triple mutant (EP61) cultures are shown in red. The *Inset* at upper right shows genome-wide mean ribosome occupancy values at nonstart AUG codons + SEM for T0 and T2.
a ribosomal protein involved in decoding (RpsQ), the master feedback repressor of methionine biosynthesis (MetJ), and a transcription factor that terminates transcription when it becomes uncoupled from translation (Rho). These three mutations exhibited specific effects on transcription and translation consistent with the hypothesis that ethanol alters the conformations of ribosomes and RNApol in ways that increase misreading, ribosome stalling, and RNApol pausing, leading to increased Rho termination when transcription and translation become uncoupled. RpsQ[E31P] increased translational fidelity and suppressed ethanol-induced misreading (Fig. 2). MetJ[ΔE91] up-regulated expression of methionine biosynthesis genes and may have partially countered an ethanol-induced stalling of ribosomes at nonstart AUG codons (Table S2, Fig. 5, and Fig. S4). Rho[L270M] reduced transcript termination at Rho-dependent terminators and contributed to amelioration of ethanol-induced premature termination on poorly translated genes (Fig. S3 and Fig. 3). Finally, we found that ethanol directly slows transcript elongation by RNA polymerase, which could increase opportunities for intragenic Rho-dependent termination (Fig. 4).

Taken together, these data indicate that multiple effects on the transcription and translation machinery are important components of cellular ethanol stress (Fig. 6). In the presence of ethanol, increased ribosome stalling (particularly at nonstart AUG codons) and increased ribosome misreading inhibit polypeptide synthesis and increase the levels of misfolded and aberrant proteins in the cell. Ethanol-induced misreading errors may exacerbate stalling and chain termination due to ribosomal proofreading after peptide bond formation (43). Slower translation and increased translational termination can decouple translation from transcription, promoting Rho-dependent termination of transcription within genes. Ethanol-induced slowing of RNApol further sensitizes transcription to termination by Rho.

The ethanol tolerance conferred by metJ mutations may reflect the inhibitory effects of ethanol on translation that are affected by methionyl-tRNA<sup>Met</sup> levels. Ethanol increases ribosome dwell time on internal AUG codons (Fig. 5), suggesting that methionyl-tRNA<sup>Met</sup> becomes limiting for protein synthesis; thus, elevated methionine levels might partially compensate for this effect. Consistent with this hypothesis, methionine supplementation or metJ deletion increased ethanol tolerance of wild-type <i>E. coli</i> grown in minimal medium (Fig. S4). Deletions and point mutations in metJ are known to increase methionine levels in <i>E. coli</i> (47, 48); the metJ[ΔE91] mutation likely causes a similar effect by elevating transcription of the <i>metABFKR</i> genes (Table S2). Interestingly, elevating methionine biosynthesis or supplementing with methionine also compensates for other stresses in <i>E. coli</i>, including acetate, organic acids, nitrosating agents, and heat shock (49–51). MetA, which catalyzes the first step in methionine biosynthesis, is known to aggregate during heat stress (51), suggesting that stress-induced MetA aggregation may reduce functional MetA and resultant methionine levels. Ethanol is a potent inducer of the heat shock response (27) and thus could similarly cause MetA aggregation, resulting in decreased methionine and methionyl-tRNA<sup>Met</sup> levels that could be partially corrected by the metJ[ΔE91] mutation. Although other compensatory effects on cell growth of elevating methionine biosynthesis remain possible, effects on translation are consistent with our detection of ethanol-induced stalling on internal Met codons.

In addition to causing toxic effects related to methionine, our data indicate that ethanol interferes with the processes by which ribosomes and RNApol produce proteins and RNA. Ethanol may alter the activities of the ribosome and RNApol through interactions at specific sites or through less-specific solute effects on macromolecular conformation. The latter possibility is attractive because both the ribosome and RNApol are multisubunit complexes whose activities require significant conformational changes during repeated cycles of chain extension and because the ethanol levels at which toxic effects occur (1-2 M) are sufficient to alter the activities of water and solutes at protein surfaces (52). Indeed, solute effects are known to alter RNApol pausing and elongation (53) in patterns and magnitude similar to those we observed for ethanol (Fig. 4). Ethanol appears to destabilize proteins by promoting exposure of hydrophobic regions (54, 55) through direct interactions (56). Ethanol destabilization of protein structure via global effects as a solute may contribute to the well-known induction of the unfolded protein response (heat-shock response) by ethanol in <i>E. coli</i> (57, 58). Ethanol-induced amino acid misincorporation to generate nonnative antibiotics such as streptomycin, coupled with previous findings, suggests that ethanol may alter the conformation of the decoding center of the small ribosomal subunit. Ribosomes select the correct aminoacyl-tRNA by coupling correct codon-anticodon pairing in the decoding center to GTP hydrolysis by elongation factor Tu (EF-Tu), leading to tRNA accommodation into the peptidyl transferase center (58). Ethanol increases ribosomal misreading in vitro (24–26) and causes changes to the ribosome footprint in a manner similar to antibiotics such as streptomycin and neomycin (59). A mutation in <i>rpsL</i>, encoding a ribosomal protein known to be important for streptomycin binding and accurate translation (512), was shown to increase ethanol tolerance in an <i>E. coli</i> <i>rho</i> mutant background (14). Additionally, ethanol tolerance is conferred on <i>E. coli</i> by overexpression (17) of RlmH, which methylates a 23S rRNA pseudouridine near the ribosomal decoding center (60), and of TruB, which catalyzes formation of pseudouridine-55 on tRNAs and is important for efficient translation of certain
codons (61). Finally, ethanol has been reported to promote the growth of streptomycin-dependent mutants of *E. coli* in the absence of streptomycin (41, 62), suggesting that ethanol, like streptomycin, may induce compensatory conformational changes in the decoding center of the ribosome. We propose that ethanol disrupts the natural conformation of the ribosomal decoding center and thus, like streptomycin, allows EF-Tu GTP hydrolysis and accommodation upon binding of noncognate aminoacyl tRNAs. An ethanol-induced rearrangement of the decoding center might also affect the propensity of the ribosome to stall and possibly its ability to recognize methionyl tRNA although other, distal effects of ethanol on the ribosome also could cause translational halting.

Our proposal that ethanol exerts its toxic effects on *E. coli* in part through direct effects on the ribosome and RNAP contrasts with previous suggestions that ethanol tolerance mutations modifying proteins involved in transcription and translation function indirectly by “rewiring” gene-expression networks. For example, increased alcohol tolerance of strains with reduced Rho activity (14, 20) or mutations in RNA polymerase subunits or the TATA-binding protein (4, 22, 63) have been attributed to altered expression of specific genes controlled by termination or initiation. Our results do not preclude the possibility that increased expression of some genes contributes to ethanol tolerance; both effects on the transcription/translation machinery and effects on gene expression may occur, and both may be important. Rather, we suggest that the potentially important and simpler explanation that compensatory mutations may help shield the ribosome and RNAP from direct effects of ethanol should not be overlooked. Indeed, some indirect effects of ethanol may also be consequences of primary effects on the ribosome or RNAP. For example, we speculate that the ethanol-induced changes in *E. coli* membrane lipid composition (10) might be caused in part by ethanol’s effects on the ribosome. Ethanol stress results in (p)pGpp production (27), likely by the RelA synthase bound to stalled ribosomes. (p)pGpp binds numerous cellular enzymes (64) and is known to inhibit the activity of the phospholipid synthesis enzyme PslB in vivo and in cell-free extracts (65, 66). Mutation of *pslB* alters fatty acid composition similarly to effects of ethanol (increased unsaturated C<sub>18:1</sub> fatty acids) (67), providing a potential link between the effects of ethanol on the ribosome and on membrane composition (10, 11) mediated by (p)pGpp. Increased (p)pGpp levels affect transcription from numerous promoters in the cell (28, 68), which could lead to other downstream effects as well. Thus, our finding that ethanol induces ribosome stalling also potentially provides a simple mechanistic explanation for ethanol stimulation of (p)pGpp production and consequent effects on multiple cellular functions as a signal of translational stress.

A key question raised by our work is whether modes of ethanol tolerance related to its direct effects on the ribosome and RNAP are broadly applicable to other solvents and other microbes. Some ethanol-tolerant mutants of *Saccharomyces cerevisiae* carry ribosomal mutations that alter antibiotic sensitivities (69), suggesting that the toxic effects of ethanol on the ribosome may operate in yeast as well as bacteria. Do naturally ethanol-tolerant species such as *S. cerevisiae* protect their ribosomes from ethanol-induced translational error and their RNAPs from ethanol-induced transcriptional slowing? Do other small-molecule solvents have ethanol-like toxic effects? The answers to these questions could have important consequences for biosynthetic engineering. If translation and transcription are widely prone to inhibition by solvents such as ethanol, then engineering microbes with resilient ribosomes and RNAPs may enhance biological production of a variety of small molecules. More generally, our results highlight the importance of considering direct effects on central dogma processes when evaluating the effects of solutes on microbes.

**Materials and Methods**

**Bacterial Strains, Media, and Growth Conditions.** All strains are derivatives of *E. coli* K12 strain MG1655 (Table 1). Strains MTA156, MTA157, and MTA160 were selected after 24 aerobic serial passages of MG1655 in M9 minimal medium (70), supplemented with MgSO<sub>4</sub> (1 mM), CaCl<sub>2</sub> (0.1 mM), and glucose (10 g/L), and increasing amounts of ethanol up to 65 g/L (Fig. 1). We isolated MTA156, MTA157, and MTA160 as clonal strains from the evolved cultures by single-colony purification. The genomes of MG1655, MTA156, MTA157, and MTA160 were sequenced at the University of Wisconsin-Madison (UW-Madison) Biotechnology Center using the Illumina HiSeq 2000 platform. Unmarked transfer of alleles between MG1655 and MTA156 was achieved by P1 transduction, first transducing in an auxotrophic marker from the Keio collection (71) linked to the desired locus and then selecting for protoporic transductants from the appropriate donor, as previously described for rho mutations (72). EP23 was constructed by P1 transduction of the metE::kan mutation from the Keio collection into MG1655. RL2739 was constructed by ρ. Red recombination of the rpsL150 allele into MG1655 after amplification from strain DH10B (40) by PCR using forward primer 5′-GGCCGCAGCCGGCGATGCG-3′ and reverse primer 5′-GGCCGCCGACGTCGATAAGA-3′.

**Fermentation Experiments.** For fermentation data shown in Fig. 1E, strains MTA376 and MTA722 were constructed by introducing the lidA4::kan allele from the Keio collection into MG1655 and EP61, respectively, by transduction, followed by removal of the kan marker by FLP recombinase (71). The ethanologenic PET cassette was expressed in each strain from plasmid pJG22 (52). Fermentative cultivations were performed in stirred flasks incubated at 37 °C in an anaerobic chamber with an atmosphere of 10% CO<sub>2</sub> + 10% H<sub>2</sub>. The medium for fermentations was synthetic com stover hydrolysate (73) supplemented with glucose (60 g/L) and xylose (30 g/L) to mimic 9% glucon loading. End product detection was done as described previously (73).

**Rho-Dependent Terminator Readthrough Analysis.** MG1655 and RL2325 cultures were grown, RNA was harvested, and RNASeq libraries were prepared as described (30). Sequencing was performed by the Joint Genome Institute using an Illumina Genome Analyzer II. Normalized RNASeq reads were summed within regions previously defined by terminator readthrough in Rho-inhibited cells (45). Fold changes in terminator readthrough were calculated by averaging the read counts for two biological replicates, and then dividing the mutant read count by the wild-type read count. Fold change could not be calculated for terminators with an average read count of zero for the wild-type samples; such terminators were removed from the final analysis. Statistical significance was tested by comparing fold changes for Rho-dependent terminators versus random sites in the genome of the same length (obtained by rotating the positions of terminator readthrough by 1 Mb).

**In Vitro Transcription Assay.** Template DNA was made from plasmid pMK110, which was constructed from pla267 (74) by inserting a region from the *E. coli* bgl operon into the SpeI site using forward primer 5′-GGGAGCCTAGTGGT-CAAGAATACGCCAGGA and reverse primer 5′-GGGAGCCTAGTGGGCGATGAGA-GCTGTAAAAT. pmk110 template DNA contains a ρ promoter followed by a 26-nucleotide C-less cassette. The linear pmk110 template for transcription reactions was generated by PCR amplification using forward primer 5′-CGTTAATATATCACGCAGGAGG and reverse primer 5′-GGATCCCTACTCTCGCATG. PCR products were electroeluted from an agarose gel and phenol extracted. Core *E. coli* RNAP and core σ<sub>70</sub> were purified as described previously (75, 76). RNAP holoenzyme (core σ<sub>70</sub> plus σ<sub>70</sub>) was prepared by incubating twofold molar excess of σ<sub>70</sub> with core for 30 min at 30 °C.

Halted elongation complexes were formed by incubating 10 nM linear pmk110 template and 15 nM RNAP holoenzyme in transcription buffer [40 mM Tris HCl (pH 8.0), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM DTT, and 5% (vol/vol) glycerol] with 150 μM ApU, 10 μM ATP and UTP, 2.5 μM GTP, and 0.37 μM (10 μL) [U<sup>32</sup>P]GTP for 10 min at 37 °C to stall complexes 26 nucleotides downstream of the transcription start site. Resumption of transcription was allowed by adding 30 μM each ATP, UTP, CTP, and GTP, 100 μg rifampicin/mL, and 0.1 U RnaseL (Promega). Ethanol was added where indicated. Samples were taken at indicated time points by mixing with an equal volume of 2X stop dye (8 M urea, 30 mM Na<sub>2</sub>EDTA, 0.05% bromophenol blue and xylene cyanol). Samples were heated for 2 min at 90 °C and separated by electrophoresis in a denaturing 6% polyacrylamide gel (9:1 acrylamide: bisacrylamide) in 7 M urea, 1.25 mM Na<sub>2</sub>EDTA, and 44 mM Tris borate (pH 8.3). Gels were exposed to a Phosphorimag screen, which was scanned using a Typhoon Phosphorimag and quantified using ImageQuant software (GE Healthcare).

Haft et al.
Densitometry profiles were generated by converting pixels from the Phosphorimage scan to transcript positions by comparison with end-labeled M6p-oligos (18). rRNA2322 using a 6-factor polynomial function. The mean transcript length was calculated from the summed products of the transcript length (in nt) times signal intensity divided by total signal intensity.

Ethanol–Antibiotic Synergy Experiments. Indicated strains (MG1655 and RIL2739) were cultured in 96-well plates (BD Falcon) in Neidhardt rich medium with 2 g glucose/L (77) supplemented with ethanol, translation inhibitors, or both. Wells were tipped with mineral oil to prevent ethanol evaporation as described (78, 79), and growth was tracked by measuring the absorbance at 595 nm in a Tecan Infinite F200 plate reader. Fitness was defined as the ratio of stressed to unstressed logarithmic growth rates.

Measurement of Translational Misreading. Cultures for misreading experiments were diluted from unstressed overnight cultures and grown to mid-logarithmic phase in aerobic tubes in Neidhardt rich medium with 2 g glucose/L (77) supplemented with 100 μg ampicillin/mL (to maintain luciferase-expressing plasmids), 0.4–0 g ETOH/L, and 10–100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Equivalent numbers of cells were harvested from each culture. Cells were lysed, and firefly luciferase (F-luc) and jellyfish luciferase (R-luc) activities were measured as described (34). Luminescence was measured using a Tecan Infinite F200 plate reader. Values are expressed as F-luc/R-luc ratios normalized to the F-luc/R-luc ratio of an isogenic strain carrying the wild-type firefly luciferase to control for any differences between cultures and differential effects of ethanol on the two enzymes.

Ribosome Profiling. Cultures were grown aerobi- cally in 1-L volumes of M9 minimal medium (70), supplemented with MgSO4 (1 mM), CaCl2 (0.1 mM), and glucose (10 g/L) in vigorously shaken (225 rpm) Fernbach flasks. Ethanol was added to 40 g of EtOH/L once A600 of cultures reached 0.3. This ethanol concentration was chosen because it allowed comparison of wild-type and mutant phenotypes under conditions in which both could grow and both exhibited growth responses to ethanol. Ribosome profiling and library generation were performed as described by Oh et al. (80), with cells harvested by sucrose gradient fractionation.

Sequencing was performed at the UW-Madison Biotechnology Center using an Illumina HiSeq 2000 set for 50-bp single-end reads. Raw reads were trimmed by 2 nt from the 5’ end (to remove any nontemplated nucleotides added by reverse transcriptase) and were then mapped using the Burrows-Wheeler Aligner (81) to the MG1655 genome (National Center for Biotechnology Information accession no. NC_000913). Reads were not trimmed to individual codons to avoid errors arising from the variation in ribosome footprint dimensions at different genomic locations (82). Signals mapping to noncoding RNA regions were removed from the dataset, and each dataset was normalized to reads per million per position before further analysis.

Data manipulation and analyses were performed with custom Perl scripts. Statistical analyses were performed using GraphPad Prism (GraphPad Software). For metagenes analyses (gene-segment analyses and codon-type analyses), pseudogenes and genes not represented in one or more datasets were excluded, leaving 3,048 genes in the “all genes” dataset. High-translational and low-translation quintiles were defined as the gene sets with highest/lowest ribosome-occupancy-to-RNA signal ratios in MG1655 before ethanol treatment. For gene-segment analyses, differences between datasets were assessed using area under the curve (rectangular midpoint approximations) for each gene in the set.

ACKNOWLEDGMENTS. We thank our Great Lakes Bioenergy Research Center collaborators and R.L. laboratory colleagues for critical reading of the manuscript. We are grateful to Gene-Wei Li and David Burkhardt for advice and assistance with ribosome profiling experiments, P. Chu for technical assistance, and Genwald Jogli for helpful discussions of ribosome decoding. This work was funded by the Department of Energy Great Lakes Bioenergy Research Center (DOE BER Office of Science Grant DE-FC02-07ER64494).

Fig. 51. Mutations in \( \text{rho} \), \( \text{metJ} \), and \( \text{rpsQ} \) cooperatively confer ethanol tolerance and increase ethanol yield. (A and B) Turbidometric densities achieved after 24 h of aerobic growth in minimal glucose medium containing 60 g ethanol/L are shown for MG1655, the evolved MTA156 mutant, and derivatives of MTA156 with wild-type alleles at the indicated loci (A) or derivatives of MG1655 with various combinations of individual mutations found in MTA156 (B). Error bars depict the values from two biological replicates. (C and D) Representative anaerobic growth curves for MG1655 and the ethanol-tolerant triple mutant (EP61) grown in minimal glucose medium in the absence (C) or presence (D) of 50 g ethanol/L. (E) Representative graph of specific ethanol yield over time for fermentation of synthetic corn stover hydrolysate (1) by MTA376 and MTA722 in stirred anaerobic flasks.

Fig. S2. The rpsL150 allele confers tolerance to ethanol. Strains MG1655 and RL2739 were cultured in the presence or absence of 20 g ethanol/L. Mean relative fitness is shown with SEM for at least three replicate cultures.

Fig. S3. Readthrough of Rho-dependent terminators in a rho[L270M] strain. The ratios of RNAseq signal for a rho[L270M] mutant (RL2325) versus wild-type (MG1655) are shown for 114 previously described regions downstream of Rho-dependent terminators (1). Bars represent the average of two biological replicates for each strain. A black horizontal line at $y = 1$ indicates the ratio observed if readthrough at a given terminator was identical in wild-type and the mutant.

Growth in the presence of 50 g EtOH/L

Fig. S4. Methionine supplementation or deletion of metJ led to improved growth in the presence of ethanol. Growth in the presence of 50 g EtOH/L of MG1655 without methionine supplementation, MG1655 supplemented with 2 mM methionine, and EP23 in the absence of methionine supplementation. Values shown are the means of two biological replicates, which differed by no more than 6% of the mean value at any point.

Table S1. Nonsynonymous mutations found in evolved strains

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTA156, MTA160</td>
<td>ispB</td>
<td>ATC→CTC</td>
<td>I32L</td>
</tr>
<tr>
<td>MTA156, MTA160</td>
<td>lptF</td>
<td>GCAGAACTG→GCACTG</td>
<td>ΔE265</td>
</tr>
<tr>
<td>MTA156, MTA160</td>
<td>metJ</td>
<td>AAAGAGATC→AAGATC</td>
<td>ΔE91</td>
</tr>
<tr>
<td>MTA156, MTA160</td>
<td>rpsQ</td>
<td>CAC→CCC</td>
<td>H31P</td>
</tr>
<tr>
<td>MTA156, MTA160</td>
<td>topA</td>
<td>CAC→CTC</td>
<td>H122L</td>
</tr>
<tr>
<td>MTA156</td>
<td>rho</td>
<td>CTG→ATG</td>
<td>L270M</td>
</tr>
<tr>
<td>MTA157</td>
<td>metJ</td>
<td>GTG→GAG</td>
<td>V33E</td>
</tr>
<tr>
<td>MTA157</td>
<td>nusA</td>
<td>CGT→GCTG</td>
<td>R258G</td>
</tr>
<tr>
<td>MTA157</td>
<td>setB</td>
<td>116-bp deletion</td>
<td>Q34R T35A P36L STOP</td>
</tr>
<tr>
<td>MTA157</td>
<td>tqsA</td>
<td>AAT→TAT</td>
<td>N234Y</td>
</tr>
<tr>
<td>MTA160</td>
<td>rho</td>
<td>CTG→CAG</td>
<td>L207Q</td>
</tr>
</tbody>
</table>

Table S2. mRNA levels of met genes reveal reduced MetJ-mediated repression in EP61

<table>
<thead>
<tr>
<th>Gene</th>
<th>Wild-type*</th>
<th>metJ rho rpsQ*</th>
<th>Fold change (mutant/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>metA</td>
<td>545 ± 220</td>
<td>784 ± 99</td>
<td>1.4</td>
</tr>
<tr>
<td>metB</td>
<td>270 ± 38</td>
<td>833 ± 4</td>
<td>3.1</td>
</tr>
<tr>
<td>metC</td>
<td>321 ± 72</td>
<td>393 ± 154</td>
<td>1.2</td>
</tr>
<tr>
<td>metE</td>
<td>12,839 ± 253</td>
<td>13,848 ± 1280</td>
<td>1.1</td>
</tr>
<tr>
<td>metF</td>
<td>1,105 ± 47</td>
<td>1,456 ± 189</td>
<td>1.3</td>
</tr>
<tr>
<td>metI</td>
<td>468 ± 183</td>
<td>954 ± 158</td>
<td>2.0</td>
</tr>
<tr>
<td>metK</td>
<td>2,501 ± 122</td>
<td>3,081 ± 278</td>
<td>1.2</td>
</tr>
<tr>
<td>metN</td>
<td>333 ± 40</td>
<td>778 ± 21</td>
<td>2.3</td>
</tr>
<tr>
<td>metQ</td>
<td>1,722 ± 315</td>
<td>1,665 ± 42</td>
<td>1.0</td>
</tr>
<tr>
<td>metR</td>
<td>287 ± 1</td>
<td>474 ± 28</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Mean total RNA signals from RNAseq experiments in RPKM (reads per kilobase per million) of two biological replicates are shown for the indicated genes before ethanol addition in MG1655 and EP61 (errors indicate total range of biological replicates). For this set of genes, which are all negatively regulated by MetJ (1), the mutant has an overall higher level of expression than wild-type (P < 0.01 by the Wilcoxon matched-pairs rank test).

Dataset S1.  

**rho**[L270M] effects on readthrough of Rho-dependent terminators

**Dataset S1**