Proline-rich antimicrobial peptides (PrAMPs) are cationic antimicrobial peptides unusual for their ability to penetrate bacterial membranes and kill cells without causing membrane permeabilization. Structural studies show that many such PrAMPs bind deep in the peptide exit channel of the ribosome, near the peptidyl transfer center. Biochemical studies of the particular synthetic PrAMP oncocin112 (Onc112) suggest that on reaching the cytoplasm, the peptide occupies its binding site prior to the transition from initiation to the elongation phase of translation, thus blocking further initiation events. We present a superresolution fluorescence microscopy study of the long-term effects of Onc112 on ribosome, elongation factor-Tu (EF-Tu), and DNA spatial distributions and diffusive properties in intact Escherichia coli cells. The new data corroborate earlier mechanistic inferences from studies in vitro. Comparisons with the diffusive behavior induced by the ribosome-binding antibiotics chloramphenicol and kasugamycin show how the specific location of each agent’s ribosomal binding site affects the long-term distribution of ribosomal species between 30S and 50S subunits versus 70S polysomes. Analysis of the single-step displacements from ribosome and EF-Tu diffusive trajectories before and after Onc112 treatment suggests that the act of codon testing of noncognate ternary complexes (TCs) at the ribosomal A-site enhances the dissociation rate of such TCs from their 70S tethers. Testing and rejection of noncognate TCs on a sub-ms timescale is essential to enable incorporation of the rare cognate amino acids into the growing peptide chain at a rate of ~20 aa/s.

Most antibiotics currently in use against bacterial infections disable a specific essential cellular mechanism, such as translation (e.g. erythromycin), transcription (rifampicin), membrane biosynthesis (penicillin), or DNA replication (ciprofloxacin) (1). As a result, localized mutations in the targeted species often develop resistance. As harmful bacteria become increasingly resistant to antibiotics, there is a critical need to develop new antibacterial agents. A promising class of compounds is the large family of antimicrobial peptides (AMPs), part of the innate immune response of a wide variety of organisms, including humans (2, 3). Many of these short (~20–50 aa long), typically cationic peptides are potent bactericidal agents that leave eukaryotic cells largely unharmed (4, 5). The first step in the attack of most cationic AMPs is the permeabilization of the membrane(s) of Gram-negative or Gram-positive species (2, 3). In general, on gaining access to the bacterial cytoplasm, AMPs subsequently disrupt a variety of cellular processes (6, 7).

Intriguingly, the subclass known as proline-rich AMPs (PrAMPs) evidently attack and kill Gram-negative bacteria such as Escherichia coli without permeabilizing the membranes (8). These cationic peptides are enriched in proline residues, which often occur in conserved patterns that also include arginine residues (9). Examples include apidaecin from bees, oncocin from milkweed bugs, and bactenecin 5 and 7 from ruminant animals (9–11). At low concentrations near the minimum inhibitory concentration (MIC), the PrAMPs primarily use a “Trojan horse” mechanism to enter the bacterial cell via the SbmA transporter or one of its homologs (9, 12). SbmA resides in the cytoplasmic membrane of some Gram-negative species and imports the highly cationic PrAMPs into the cytoplasm using the proton motive force. Once they have entered the cytoplasm, the PrAMPs target 70S ribosomes. No homolog of the SbmA transporter occurs in eukaryotic cells. In addition, multiple studies have shown that PrAMPs are potent mostly against the bacterial 70S ribosomes and not the eukaryotic ribosomes (13, 14). Thus, PrAMPs have low cytotoxicity toward eukaryotic cells. This makes PrAMPs an attractive class of antibacterial agents for further refinement (11).

One such natural PrAMP is oncocin. Specific amino acid residues of oncocin have recently been modified to increase its potency, giving rise to the synthetic oncocin112 (Onc112; sequence VDKPPYLPRPRPPrIYNr-NH₂, in which r denotes D-arginine; net charge +6) (15, 16). In 2015, two laboratory-published X-ray crystallography structures of Onc112 bound to the 70S ribosome in complex with a short mRNA and with a deacylated tRNA^{Met} or tRNA^{Met} bound at the P-site (17, 18). The structures showed that Onc112 binds deep within the peptide exit channel and extends into the peptidyl transferase center, where it overlaps what would be the binding site of an incoming aminoacyl-tRNA (aa-tRNA) at the ribosomal A-site (Fig. 1a). From this structure and from biochemical experiments (18, 19), it was inferred that Onc112 blocks the transition of the 70S ribosome from the initiation complex to the peptide elongation phase of protein synthesis. The suggestion is that Onc112 enters the ribosome through an empty peptide exit channel; i.e., Onc112 must bind before elongation has begun to fill the exit channel with growing peptide and thus block the Onc112 binding site (18). Ribosomes bearing Onc112 stall at the initiation site and cannot transition to peptide elongation. Additional
experiments using a short, dicistronic mRNA suggested that binding of Onc112 also destabilizes the initiation complex itself, presumably because of spatial overlap between Onc112 and the fMet moiety of the fMet-tRNA<sub>fMet</sub> bound in the P-site (18). Ribosomes can still assemble as 70S initiation complexes on the mRNA, but with decreased stability.

This is somewhat reminiscent of the behavior of certain ribosome-targeting antibiotics such as chloramphenicol (Cam), kasugamycin (Ksg), and many others. Cam binds the 70S ribosome at the A-site crevice near the exit channel, hindering the binding of an incoming aa-tRNA to the A-site (Fig. 1a) (20). Unlike Onc112, Cam can bind to elongating ribosomes, not only to initiation complexes (21). Cam potentially blocks elongation by all 70S ribosomes. In another example, Ksg binds to the mRNA path within the 30S subunit, overlapping both the E-site and P-site (Fig. 1a) (22, 23). Ksg prevents binding of fMet-tRNA<sub>fMet</sub> and also blocks the E-site to prevent elongation. Thus, Ksg halts the initiation step and prevents the onset of elongation (22, 23).

We have used fluorescence microscopy to study in real time the sequence of events during the attack of AMPs on single, live E. coli cells (24, 25). Different assays can determine the time of onset of outer membrane permeabilization, of cytoplasmic membrane permeabilization (26), of the halting of growth, of the induction of oxidative stress (27), and of alterations in the spatial distributions and diffusive properties of a variety of cytoplasmic components (6). We have also previously studied the dynamics of different components of the translation machinery (28, 29), specifically the elongation cycle, like EF-Tu and ribosomes in live E. coli cells using superresolution fluorescence microscopy (30, 31). We have shown using diffusion analysis that multiple EF-Tus can bind to the C-terminal domain of the L7/L12 stalk of ribosomes, thereby increasing their local concentration to expedite the search for a cognate ternary complex (TC). We have also measured the upper limit of the time spent by a near-cognate or non-cognate ternary complex bound to the L7/L12 stalk, which is ~1 ms (30). Two-state diffusion analysis of ribosomes show that the majority of them remain in a polysome state (30). In this study, we quantitatively characterize the long-term effects of Onc112 on the spatial distributions of ribosomes, EF-Tu, and the chromosomal DNA in intact E. coli cells. We also compare the effects of Onc112, Cam, and Ksg on the diffusive behavior of the same cytoplasmic components. The results help to corroborate earlier mechanistic inferences from in vitro studies and provide new biophysical insight into their effects on the overall condition of the E. coli cytoplasm.

Onc112 and Ksg, both of which block transitions to the elongation phase of translation, have very similar effects on ribosome diffusion, increasing the average diffusion coefficient by a factor of 2. Cam, which can halt translation by 70S ribosomes during the elongation phase, has a minor effect on the average ribosome diffusion coefficient. All three treatments decrease the average EF-Tu diffusion coefficient by a factor of 1.3–1.6. These results correlate well with information from the earlier structural studies of the Onc112-, Ksg-, and Cam-specific binding sites within the ribosome (20, 22). In addition, analysis of the single-step displacement probability distributions from ribosome and EF-Tu diffusive trajectories suggests that the act of codon testing of noncognate TCs at the ribosomal A-site enhances the dissociation rate of such TCs from their L7/L12 tethers. This enables sufficiently rapid codon testing to keep up with the very fast elongation rate of ribosomes in good growth conditions. Based on our data, we have proposed a scheme of how Onc112 affects the translation process (Fig. 1b) and how this mechanism differs from that of Cam (Fig. 1c).

**Results**

**Effects of Onc112 on cell growth**

The MIC of Onc112 over 6 h in EZ rich defined medium (EZRDM) at 30°C is 1 μM (Fig. S7) (16). To directly observe slowing and halting of growth, we plated WT VH1000 cells on a poly-L-lysine–coated coverslip enclosed by a microfluidic chamber. Such cells grow normally in a flow of fresh, aerated EZRDM. At t = 0, we initiated flow of a constant concentration of Onc112 in EZRDM and monitored cell length versus time, determined from phase contrast images by the program Fiji.
Onc112 effects on translation machinery in live E. coli

Figure 2. The effect of Onc112 on cell length. a, average cell length change versus time for VH1000 WT cells after the onset of flow of different concentrations of Onc112. Cell lengths obtained from phase contrast images are normalized to the length at time 0: \( \frac{L(t)}{L_0} \). Onc112 was injected at 1 min. Effects of Onc112 become noticeable at \( t \sim 5 \) min after injection. b, cell length distributions before and after Onc112 treatment. The cell cultures were treated with 20 \( \mu \mathrm{M} \) of Onc112 for \( 35 \) min before imaging. c and d, examples of phase contrast images before and after 20 \( \mu \mathrm{M} \) Onc112 treatment, respectively.

(32). In Fig. 2a, we plot \( \frac{L(t)}{L_0} \), the cell length as a function of time normalized to the length at \( t = 0 \) and averaged over 10–23 cells for different concentrations of Onc112. For all concentrations, the plots begin to curve downward at \( t \sim 5 \) min after the onset of Onc112 flow. The higher the concentration of Onc112, the sooner cell length begins to plateau. For 5 \( \mu \mathrm{M} \) and higher concentrations, the effect on cell growth is similar.

To test for evidence of outer membrane disruption by Onc112 in our conditions, we applied 20 \( \mu \mathrm{M} \) solution of Onc112 to cells expressing GFP with a tag directing transport to the periplasm (strain JCW10, Table S1). We have used 20 \( \mu \mathrm{M} \) solution of Onc112 (20 \( \times \) MIC) for most of our microscope-based experiments. Such a high concentration was used to ensure that essentially all of the cells were affected by Onc112 prior to observation by fluorescence microscopy. Sytox Orange was also added to the flowing medium with a final concentration of 5 nM. In our standard assay for cell growth and for outer and cytoplasmic membrane permeabilization to GFP and Sytox Orange (26), phase contrast and green and red fluorescence images were captured sequentially in time, with a cycle time of 12 s. We saw no evidence of membrane permeabilization to GFP and Sytox Orange, at least up to Onc112 concentrations of 20 \( \mu \mathrm{M} \) over a period of 55 min (Fig. S1). The phase contrast images remain stable at least up to Onc112 concentrations of 20 \( \mu \mathrm{M} \) over a period of 45 min, indicating little or no loss of cytoplasmic components. For multiple AMPs in the past, a sharp decrease in cell length with the injection of the peptide has been observed (8, 33). This shrinkage is correlated to the disruption of the bacterial membranes by the AMP. There is no evidence of such cell shrinkage or of membrane disruption in our conditions.

Spatial distribution of ribosomes, EF-Tu, and DNA after Onc112 treatment

For these experiments, cells were grown to the exponential phase (optical density (OD) \( \sim 0.2–0.4 \)) in EZRDM in culture tubes at 30 °C. At that point we added Onc112 to 20 \( \mu \mathrm{M} \) (20 \( \times \) MIC) final concentration and incubated the culture for an additional 30–40 min before plating the cells for fluorescence microscopy. The modified VH1000 strains of E. coli and their labeling schemes are described in Table S1. As before (28, 30, 31, 34), to locate and track single copies of ribosomal species (MSG196 strain, S2-mEos2 labeling) and of EF-Tu (tufAB strain, mEos2 labeling), we used superresolution fluorescence microscopy (see “Experimental procedures”). The gene modifications are made on the chromosome, so that all copies of the protein of interest carry the label. The doubling times of WT, MSG196, and tufAB strains in EZRDM at 30 °C are 45 ± 2 min, 49 ± 2 min, and 60 ± 3 min, respectively. Labeling of the S2 protein has only a minor effect on cell growth, whereas labeling of the protein EF-Tu causes a moderate growth defect of \( \sim 33\% \). Fig. 2b compares the length distribution of cells growing normally in EZRDM with that of cells treated with 20 \( \mu \mathrm{M} \) Onc112 for 30–40 min. The distribution of the treated cells is shifted toward smaller lengths, consistent with slowing and then halting of growth.

To detect rapidly diffusing species on the same footing as slowly diffusing species, we imaged single molecules at 2 ms/frame. In each camera frame we maintained a low density of photoactivated mEos2, with zero, one, or two copies/cell. The resulting single-molecule trajectories are short, averaging only about three camera frames. For untreated cells in exponential growth, the S2 protein is essentially always incorporated into a complete 30S ribosomal subunit. However, the 30S subunits we track may occur as freely diffusing 30S copies searching for translation initiation sites or as 30S subunits incorporated into translating 70S ribosomes, typically part of a 70S-polysome chain (28, 34). In earlier work on cells in exponential growth, we analyzed EF-Tu trajectories as arising from a binary combination of slowly diffusing, ribosome-bound copies and rapidly diffusing copies attributed to free EF-Tu or EF-Tu bound within TCs (30).

Examples of ribosome and EF-Tu trajectories using the same labeling schemes have been shown previously (30). In Fig. 3, we compare the cell-averaged ribosome and EF-Tu spatial distributions in untreated, exponentially growing cells (length ranges 3–4 \( \mu \mathrm{m} \) and 4–5 \( \mu \mathrm{m} \)) with those in cells treated with 20 \( \mu \mathrm{M} \) Onc112 for 30–40 min (length range 3–4 \( \mu \mathrm{m} \)). The average length of untreated cells was around 4.5 \( \mu \mathrm{m} \) (Fig. 2a), so both length ranges were included to have an unbiased comparison with the Onc112-treated cells. The distributions shown are obtained by averaging distributions from multiple cells in the
The number of cells \((n)\) used in each case is reported below each distribution. As observed before (28, 34), in normal exponential growth with 45-min doubling time most cells exhibit three axially distinct ribosome-rich regions (Fig. 3, a and b). In this condition, the chromosomal DNA is known to segregate into two lobes that sit in the valleys between the ribosome peaks (34). A histogram of the projected axial positions scaled to each cell length shows the three ribosome peaks more quantitatively (Fig. 3, g and h). The EF-Tu distribution may also have three axial peaks (Fig. 3, d and e), but the dips between the peaks are not nearly as pronounced as those for ribosomes. This is consistent with only part of the EF-Tu population being ribosome-bound at a given moment. The spatial distributions after Onc112 treatment are markedly different. After treatment, the ribosomes concentrated in the two endcaps (Fig. 3c). The EF-Tu distribution again roughly mimics that of the ribosomes, but the dip between nominal EF-Tu peaks is even less pronounced (Fig. 3, f and i).

Our next goal was to image ribosomes and the chromosomal DNA in single cells after Onc112 treatment to further characterize the degree of DNA-ribosome segregation. For these widefield fluorescence microscopy experiments, we used strain MSG192 (Table S1) in which the ribosomal S2 protein is tagged with enhanced YFP in the VH1000 background. This enables co-imaging of the 30S ribosomal subunit using its YFP tag and the chromosomal DNA using the DNA stain Sytox Orange. We studied 89 cells in this way; two representative single-cell images and their axially projected ribosome and DNA linescans are shown in Fig. 4, a and b. In all cells, the ribosome linescans again show two peaks at the endcaps. All the DNA linescans show a broad, centrally located peak, typically with a small dip at the cell center. There is little or no DNA in the endcaps, where DNA-ribosome segregation is strong. The small dip in the DNA axial distribution suggests the presence of two DNA lobes that are not well separated from each other. More pronounced dips occur more often in longer cells. 58 of the 89 cells (~65%) have this dip. In all cases, the position of the central dip in the DNA linescan corresponds to that of a small third peak in the ribosome linescan. In widefield images of untreated cells under the same growth conditions, we observed substantially more complete spatial segregation of the two DNA lobes as shown in Fig. 4c, and this is consistent with what we observed previously (34). Two complete fields of view for Onc112 treated and untreated cells are provided in Fig. S5 and Fig. S6, respectively.

**Diffusion of ribosomes and EF-Tu after Onc112, Cam, and Ksg treatment**

The antimicrobial peptide Onc112 and the bactericidal drugs Cam and Ksg are all known to halt translation by binding to 70S ribosomes, but the binding modes differ (17, 18, 20, 22). Next, we compared the average diffusive motion of the ribosomal species and the EF-Tu species in untreated cells in exponential growth and in cells treated by Onc112, Cam, and Ksg. The drug treatments used 300 μg/ml (20×MIC) Cam for ~35 min or 5 mg/ml (30×MIC) Ksg for ~35 min, similar to the Onc112 treatment of 20×MIC for ~35 min. These concentrations were shown to be effective against the entire poly-some population in vivo (21). To characterize the average diffusion coefficient of a species, we formed trajectories that
tracked the centroid of each single-molecule image across consecutive 2-ms camera frames. We analyzed only trajectories that survived at least seven camera frames (six steps) and truncated the longer trajectories at the seventh frame. A mean-square displacement (MSD) versus lag time plot MSD(\(t\)) was formed by measuring the square of the displacement over the lag times \(t = 1\) step, 2 steps, etc., and averaging over all molecules in all cells. For free Brownian diffusion in two dimensions, MSD(\(t\)) = 4\(D_{\text{mean}}t\) (35). We used the initial slope of the plot (first two data points) to provide an estimate of the mean diffusion coefficient, \(D_{\text{mean}}\) and the \(y\)-intercept to provide an estimate of the average localization error \(s\) (36). Only the first two points were chosen because the data becomes increasingly noisy for later points due to less averaging (36). Such noise can affect the shape of the MSD plot.

MSD(\(\tau\)) plots for ribosomes over 12 ms are shown in Fig. 5a and results are included in Table 1. The downward curvature is likely due to true subdiffusion rather than confinement effects on the short timescale of the observations. For 2 ms/frame

Figure 4. DNA and ribosome distribution using widefield fluorescence, before and after Onc112 treatment. Left column, phase contrast images of the cell and widefield fluorescence images of DNA (Sytox Orange staining) and ribosomes (S2-enhanced YFP labeling) for two different cells, (a) and (b), treated with Onc112 for \(-35\) min, and for an untreated cell (c). The effect of Onc112 treatment in different cells of different lengths is represented in (a) and (b). Right column, axial projections of the widefield fluorescence intensities.

Figure 5. Mean-square displacement plot of ribosome and EF-Tu under different drug treatment. a, MSD(\(\tau\)) plots for ribosomes labeled with the photoswitchable protein mEos2 under different conditions. Average diffusion coefficients \(D_{\text{mean}}\) are obtained from the slope of the line through the first two data points as shown. Results are collected in Table 1, b, as in panel A, MSD(\(\tau\)) plots for EF-Tu labeled with mEos2. See text and Table 1 for average diffusion coefficients \(D_{\text{mean}}\). There is some degree of subdiffusion observed for both ribosomes and EF-Tu even at a 2-ms timescale.
imaging of ribosomes in untreated, exponentially growing *E. coli*, we obtained $D_{\text{mean}} = 0.40 \pm 0.10 \, \mu m^2/s$ (30). This is reasonably consistent with a smooth extension of the strongly curved MSD plots reported previously over substantially longer timescales (37). Onc112 treatment and Ksg treatment increased $D_{\text{mean}}$ to 0.74 $\pm 0.09 \, \mu m^2/s$ (by 1.9×) and to 0.79 $\pm 0.21 \, \mu m^2/s$ (by 2.0×), respectively. Cam treatment yielded $D_{\text{mean}} = 0.48 \pm 0.11 \, \mu m^2/s$, only 1.2 times the result in normal growth.

In an attempt to resolve the ribosomal diffusive motion into contributions from two sub-populations, the same trajectories in untreated cells and in cells after Onc112 treatment were divided into individual steps of 2-ms duration, providing the distribution of apparent single-step displacements of ribosomes $P_{\text{ribo}}(r)$ (Fig. 6). In Fig. S2, we directly compare the two $P_{\text{ribo}}(r)$ curves; it is clear that ribosome diffusion is faster for Onc112-treated cells, consistent with the MSD($r$) plots. As before (30), we modeled each $P_{\text{ribo}}(r)$ distribution using a static two-state model that assumes that the displacements arise from a composite of a slowly diffusing population and a rapidly diffusing population that do not exchange on the 2-ms imaging timescale. In *E. coli*, the size of polysomes can vary a lot, which will also affect their diffusion coefficient. Thus, ribosomes can have more than two diffusive states. But in our experimental condition, because of localization error broadening, it is not possible to distinguish between the slow polysomal states. Thus, we treat all the polysomal states as the same (represented by $D_{\text{slow}}$) in our two-state diffusion analysis. The numerical model includes confinement effects and localization uncertainty (see “Experimental procedures”) (30). The fitting parameters are the diffusion coefficient of the slow population $D_{\text{slow}}$, the diffusion coefficient of the fast population $D_{\text{fast}}$ and the fraction of slow copies $f_{\text{slow}}$ by subtraction, $f_{\text{fast}} = 1 - f_{\text{slow}}$. For ribosomes in untreated cells the best-fit values are $D_{\text{slow}} = 0.10 \pm 0.10 \, \mu m^2/s$, $f_{\text{slow}} = 0.70 \pm 0.05$, $D_{\text{fast}} = 1.2 \pm 0.5 \, \mu m^2/s$, and $f_{\text{fast}} = 0.30 \pm 0.05$, with reduced chi-square ($\chi^2$) = 0.67 (30). For Onc112 treated cells, the best-fit values are $D_{\text{slow}} = 0.25 \pm 0.1 \, \mu m^2/s$, $f_{\text{slow}} = 0.46 \pm 0.05$, $D_{\text{fast}} = 1.3 \pm 0.2 \, \mu m^2/s$, and $f_{\text{fast}} = 0.54 \pm 0.05$, with $\chi^2 = 1.1$. The $D_{\text{slow}}$ represents the diffusion of bulky polysomes and the $D_{\text{fast}}$ represents the diffusion of free 30S subunits. These results are collected in Table 1. $P_{\text{ribo}}(r)$ for Onc112-treated cells differs from that of untreated cells primarily because of a shift in fractional population from the slow state to the fast state. The underlying diffusion coefficients remain sensibly constant.

The same MSD and one-step distribution analyses were applied to EF-Tu trajectories obtained at 2 ms/frame from untreated cells and from cells treated with Onc112, Cam, and Ksg. The MSD($r$) plots in Fig. 5b show that on average, EF-Tu diffuses faster in untreated cells than in cells treated with Onc112, Cam, or Ksg. The latter three plots are quite similar in slope. There is again downward curvature. The numerical results from the first two points are $D_{\text{mean}} = 2.02 \pm 0.19 \, \mu m^2/s$ for the untreated cells (30), $D_{\text{mean}} = 1.25 \pm 0.22 \, \mu m^2/s$ after Onc112 treatment (1.6× smaller than in untreated cells), $D_{\text{mean}} = 1.39 \pm 0.32 \, \mu m^2/s$ after Cam treatment (1.5× smaller), and $D_{\text{mean}} = 1.52 \pm 0.26 \, \mu m^2/s$ after Ksg treatment (1.3× smaller).

### Table 1

| Species                        | $D_{\text{mean}}$ ($\mu m^2/s$) | $f_{\text{slow}}$ | $D_{\text{slow}}$ ($\mu m^2/s$) | $D_{\text{fast}}$ ($\mu m^2/s$) | $\chi^2$ |
|-------------------------------|---------------------------------|-------------------|---------------------------------|---------------------------------|----------|
| Ribosome (untreated) (30)      | $0.40 \pm 0.10$                 | $0.70 \pm 0.05$   | $0.1 \pm 0.1$                  | $1.2 \pm 0.5$                  | 0.67     |
| Ribosome (Onc112 treatment)    | $0.74 \pm 0.09$                 | $0.46 \pm 0.05$   | $0.25 \pm 0.10$                | $1.3 \pm 0.2$                  | 1.1      |
| Ribosome (Cam treatment)       | $0.48 \pm 0.11$                 | -                 | -                               | -                               | -        |
| Ribosome (Ksg treatment)       | $0.79 \pm 0.21$                 | -                 | -                               | -                               | -        |
| EF-Tu (untreated) (30)         | $2.02 \pm 0.10$                 | $0.60 \pm 0.05$   | $1.0 \pm 0.1$                  | $4.9 \pm 1.2$                  | 1.24     |
| EF-Tu (Onc112 treatment)       | $1.25 \pm 0.22$                 | $0.72 \pm 0.05$   | $0.5 \pm 0.1$                  | $5.0 \pm 0.14$                 | 1.43     |
| EF-Tu (Cam treatment)          | $1.39 \pm 0.32$                 | $0.72 \pm 0.05$   | $0.6 \pm 0.2$                  | $3.9 \pm 0.13$                 | 1.38     |
| EF-Tu (Ksg treatment)          | $1.52 \pm 0.26$                 | -                 | -                               | -                               | -        |

*All are in VH1000 background, grown in EZRDM at 30°C. See Table S1.

* From initial slope of MSD plots (Fig. 5).

* Best-fit parameters from two-state fitting of $P(r)$ histograms for ribosomes and for EF-Tu (Fig. 6 and Fig. 7). Fractional population of the fast state is $f_{\text{fast}} = (1 - f_{\text{slow}})$. $\chi^2$ is the reduced chi-square statistic for the best-fit.

**Onc112 effects on translation machinery in live *E. coli***

![Figure 6. Single-step displacement distribution of ribosome.](image-url)
Onc112 effects on translation machinery in live E. coli

Figure 7. Single-step displacement distribution of EF-Tu. Single-step displacement distributions $P_{EF-Tu}(r)$ from single-molecule trajectories (2 ms/ frame) of EF-Tu–mEos2. Also shown are least-squares best-fits from a static, two-state model. a, normal growth conditions. b, after ~35 min of treatment with 20 μM of Onc112. c, after ~35 min of treatment with 300 μg/ml of Cam.

The single-step distributions $P_{EF-Tu}(r)$ for untreated cells and for cells treated with Onc112 and with Cam are compared in Fig. 7. These curves were fit to the same static two-state model used for ribosomes. For EF-Tu in untreated cells, the best-fit results are $D_{\text{slow}} = 1.00 \pm 0.20 \, \mu m^2/s, f_{\text{slow}} = 0.60 \pm 0.05, D_{\text{fast}} = 4.9 \pm 1.2 \, \mu m^2/s, and f_{\text{fast}} = 0.40 \pm 0.05$, with $\chi^2 = 1.2$ (Table 1) (30). The $D_{\text{slow}}$ represents a fast exchanging state of TC transi-
ently binding to the ribosomal L7/L12 stalk. A value closer to the polynomal diffusion coefficient will indicate slowdown of the transient exchange of TC between ribosome-bound and freely diffusive states. The $D_{\text{fast}}$ represents freely diffusing TC in the E. coli cytoplasm. Consistent with the MSD plots, the two-state fits after Onc112 treatment and after Cam treatment are very similar. For EF-Tu in Onc112-treated cells, the best-fit results are $D_{\text{slow}} = 0.50 \pm 0.10 \, \mu m^2/s, f_{\text{slow}} = 0.72 \pm 0.05, D_{\text{fast}} = 5.0 \pm 1.4 \, \mu m^2/s, and f_{\text{fast}} = 0.28 \pm 0.05$, with $\chi^2 = 1.4$ (Table 1). Compared with untreated cells, Onc112 decreased $D_{\text{slow}}$ by a factor of 2.0 and mildly increased $f_{\text{slow}}$ as well. The two-state results for Cam-treated cells are quite similar to the results for Onc112-treated cells (Table 1). We obtained $D_{\text{slow}} = 0.60 \pm 0.2 \, \mu m^2/s, f_{\text{slow}} = 0.72 \pm 0.05, D_{\text{fast}} = 3.9 \pm 1.3 \, \mu m^2/s, and f_{\text{fast}} = 0.28 \pm 0.05$, with $\chi^2 = 1.4$. Finally, $P_{EF-Tu}(r)$ after Ksg treatment closely mimicked the curves after Onc112 and Cam treatment (Fig. S3). This is consistent with the MSD plots. These diffusion results are interpreted under “Discussion”. We also tracked Kaede (110 KDa) as a control, which is a free fluorescent protein and is not supposed to bind to anything in the E. coli cytoplasm (38). The diffusion of Kaede gave us an estimate of how a freely diffusing fluorophore of similar size can diffuse under untreated and Onc112-treated conditions. The $D_{\text{Mean}}$ of Kaede changed from $5.00 \pm 0.09 \, \mu m^2/s$ for untreated cells to $3.82 \pm 0.25 \, \mu m^2/s$ for Onc112 treated cells (Fig. S4). This change falls within 1 $\sigma$ of our EF-Tu $D_{\text{fast}}$ fitting error.

Discussion

We have presented a substantial body of quantitative information comparing the long-term (~35 min) effects of the PrAMP Onc112 and the antibiotics Cam and Ksg on the diffusive behavior of several important cytoplasmic components. Onc112 shortens the average cell length (Fig. 2b), redistributes the DNA and the ribosomal and EF-Tu species (Fig. 3) while maintaining strong DNA-ribosome segregation (Fig. 4), increases the average diffusion coefficient of ribosomal species (Fig. 5a), and decreases the average diffusion coefficient of EF-Tu (Fig. 5b). The effects of Ksg are quantitatively similar to those of Onc112 (Fig. 5). Cam treatment has a minor effect on average ribosome diffusion but slows EF-Tu average diffusion to a similar degree as Onc112 and Ksg (Fig. 5). Here we use structural information for the Onc112, Cam, and Ksg binding sites on the ribosome to help understand the observed diffusive behavior in intact E. coli cells.

As depicted in Fig. 1a, Onc112 and Ksg bind in the peptide exit tunnel and in the mRNA path, respectively (18, 22). Both species evidently find their binding sites prior to the onset of the elongation phase of translation (9, 22). Once bound, they prevent further initiation events while allowing already elongating 70S ribosomes to continue making protein (9). For monocistronic genes, such 70S ribosomes will produce complete protein copies on a timescale of ~30 s, fall off the mRNA, and be recycled as 30S and 50S subunits. For polycistronic genes, which are fairly commonplace in E. coli, on completion of an initial protein product the 70S ribosome will either fall off the mRNA or search diffusively trying to find the next downstream Shine-Dalgarno sequence in a process termed “70S-scanning.
initiation” (39, 40). If there is no drug molecule blocking initiation of that gene’s elongation cycle, 70S may then go on to produce the next protein prescribed by the mRNA sequence. In any event, after 20–30 min of Onc112 or Ksg treatment, we would anticipate a net conversion of part of the normal population of “dense 70S polysomes” to free 30S and 50S subunits and perhaps also to “sparse 70S polysomes”, as depicted for Onc112 in Fig. 1b. The subunits will diffuse faster than the dense polysomes; the sparse polysomes probably also diffuse faster than dense polysomes. Accordingly, we observe that the average diffusion coefficient of ribosomal species increases substantially in Fig. 5 fusion coefficient of ribosomal species increases substantially dense polysomes. The subunits will diffuse faster than the dense polysomes; the sparse polysomes probably also diffuse faster than dense polysomes. Accordingly, we observe that the average diffusion coefficient of ribosomal species increases substantially after treatment with Onc112 or Ksg (Fig. 5a). In addition, the two-state decomposition of \( P_{\text{rib}}(r) \) (Fig. 6, Table 1) indicates that the primary effect of Onc112 is to increase the fractional population of the more-rapidly diffusing ribosomal species (presumably 30S subunits), again in accord with the results in Fig. 1b. We suggest that Onc112 and Ksg have very similar effects on ribosome diffusion because they alter the composition of ribosomal species in much the same way.

In contrast, Cam can bind deep within the A-site crevice of all elongating 70S ribosomes and halt translation by blocking accommodation of the next codon-matching aa-tRNA within the peptidyl transferase center (20). As before (21), we suggest that many dense 70S polysomes are retained long-term after Cam treatment, as depicted in Fig. 1b. As a result, \( D_{\text{mean}} \) for ribosomes is changed very little by Cam.

For EF-Tu, we show that before or after Onc112 treatment, the spatial distribution mimics that of the ribosomal species to a degree, but the axial peaks and valleys are less well-defined (Fig. 3). This is consistent with substantial binding of EF-Tu–containing species to those ribosomal species that segregate most strongly from DNA, presumably the remaining 70S polysomes. After all three treatments (Onc112, Ksg, and Cam), the mean diffusion coefficient of EF-Tu–containing species decreased by a factor of 1.3–1.6. The two-state decompositions of \( P_{\text{EF-Tu}}(r) \) in untreated cells and in cells treated with Onc112 and with Cam indicated quantitatively similar effects of Onc112 and Cam. Both drugs increased the fraction of the slowly diffusing population (from 0.60 ± 0.05 for untreated cells to 0.72 ± 0.05 for treated cells) while decreasing \( D_{\text{slow}} \) (from 1.0 ± 0.2 \( \mu \)m\(^2\)/s to 0.5 ± 0.1 \( \mu \)m\(^2\)/s for Onc112 and 0.6 ± 0.2 \( \mu \)m\(^2\)/s for Cam, respectively). Importantly, for both treated and untreated cells, \( D_{\text{slow}} \) for EF-Tu was significantly larger than \( D_{\text{slow}} \) for ribosomes in the corresponding condition (0.10 ± 0.10 \( \mu \)m\(^2\)/s for untreated, 0.25 ± 0.10 \( \mu \)m\(^2\)/s after Onc112 treatment) (Fig. 6).

Initial binding of TCs to the 70S ribosome occurs on any of four L7/L12 binding sites flexibly tethered to the 50S subunit near the A-site (Fig. 1a) (41–43), where the aa-tRNA within each TC is tested for a codon match. For a given mRNA codon poised at the 30S decoding site, the chance that a particular TC carries a cognate (completely matching) aa-tRNA anticodon is roughly 1 in 40 (30). Although the vast majority of aa-tRNAs fail the codon-matching test, in good growth conditions the average elongation rate can be as fast as ~20 aa/s (~50 ms per complete elongation cycle) (44, 45). This implies very rapid codon testing on a timescale of ~1 ms or even less. Therefore, testing and dissociation of noncognate and near-cognate TCs from their L7/L12 tethers must occur rapidly on the 2-ms timescale between camera frames. Accordingly, in our earlier work on untreated cells, we suggested that the best-fit value of \( D_{\text{slow}} \) for EF-Tu was in fact a weighted average over slower diffusion of TCs bound to 70S ribosomes and much faster, free diffusion of unbound TCs. In this interpretation, the smaller the value of \( D_{\text{slow}} \) for EF-Tu, the longer the dwell time that each noncognate TC spends bound to its L7/L12 tether prior to dissociation. Our earlier work indicated that the four L7/L12 tethers are essentially saturated with TC binders during fast growth. Presumably this maintains a steady supply of nearby TCs that can present themselves for codon testing very quickly each time the A-site becomes available after rejection of a noncognate TC.

Now we applied this same idea to EF-Tu diffusion after Onc112 or Cam treatment. Both treatments resulted in significantly smaller values of \( D_{\text{slow}} \) for EF-Tu compared with untreated cells. The data thus suggest that noncognate TCs (the vast majority) bind to L7/L12 for a substantially longer time period when codon testing at the A-site is blocked by the peptide or antibiotic. In other words, the interaction of a tethered, noncognate TC with the A-site during the codon-testing event somehow stimulates faster dissociation from L7/L12 than would occur for TC dissociation from an isolated L7/L12 binding site. This is a qualitative inference only. We could be more quantitative if we knew the ratio between EF-Tu and ribosome copy numbers and the fraction of ribosomes occurring as 70S after treatment with each antibacterial agent. This information is available in our earlier studies of normally growing cells (30, 31).

Rodnina, Lipowsky, and co-workers (46–48) have developed a detailed kinetics scheme for the branched, multi-step processes involved in TC binding, selection of cognate aa-tRNAs, and elongation of the growing peptide chain. Their scheme includes codon-independent rates for TC binding and unbinding to L7/L12 tethers \((k_1\text{ and } k_{-1}, \text{ respectively, in (48)}\) and \(k_{\text{on}}\) and \(k_{\text{off}}\), respectively, in (46). Our suggestion that the interaction of the tethered TC codon with the A-site enhances the dissociation rate of noncognate and near-cognate TCs from L7/L12 lends a slightly different meaning to \( k_1 \) (or \( k_{\text{on}} \)) in normal growth conditions, dissociation of noncognate TCs from L7/L12 would now be coupled to unfavorable interaction of the TC with the A-site. This is a significant step in the entire cycle that has remained elusive till now because of its fast nature.

We would also like to draw some similarity with a recent study from the Blanchard Laboratory (49). They have shown using a three-color FRET study that conformational changes occurring during the accommodation step for mostly cognate tRNAs is important for EF-Tu to leave the ribosomes. They have shown using multiple antibiotics treatment that when the accommodation step is hindered, the release of EF-Tu–GDP is slowed down significantly. This study provides important insights into what determines EF-Tu–GDP release from the ribosomes after GTP hydrolysis. In contrast, we observed mostly non- or near-cognate TCs, which do not reach the accommodation step and are rejected during the initial selection process. Our data indicate that a similar conformational change is required during this tRNA testing step for the entire TC release. This conformational change might occur after the
initial mRNA-tRNA mismatch. Because of the extremely short lifetime of this testing step, no structural detail has been available to date. We would also like to state that our inference is based on our diffusion data fitting and further structural studies are required to fully understand this process.

**Experimental procedures**

**Bacterial strains**

Most of the strains used in this work (Table S1) are modifications of the VH1000 background strain. The tufAB strain contains a C terminus fusion of the photoconvertible protein mEos2 to the tufA and tufB genes, both of which express identical copies of EF-Tu. The MSG196 and MSG192 strains contain a C terminus fusion of mEos2 and YFP, respectively, to the S2 ribosomal protein. The JCW154 strain is in the MG1655 background. In this strain the Right2 locus of the chromosome is fused with a tandem array of parS sites. This strain also contains a plasmid that expresses ParB protein fused with GFP. The construction details are given in our previous papers (27, 50). The strain JCW10 produces the protein GFP–trimethylamine-N-oxide reductase from a plasmid pW1. The appended trimethylamine-N-oxide reductase signaling sequence triggers export of GFP to the periplasm by the twin-arginine transport system (26). The description of the strains used and their corresponding doubling times are given in Table S1.

**Cell growth and preparation for imaging**

The cells were grown in a water bath shaker (New Brunswick Innova 3000, Eppendorf) maintained at 200 rpm and 30 °C. Bulk cultures were harvested from frozen glycerol stock solution and were grown in EZRDM overnight until they reached stationary phase. It is a MOPS buffer–based solution at pH = 7.4, supplemented with metal ions (M2130, Teknova), glucose (2 mg/ml), amino acids and vitamins (M2104, Teknova), nitrogenous bases (M2103, Teknova), 1.32 mM K2HPO4, and 76 mM NaCl. On the following day, the stationary phase culture was divided into subcultures with at least 100-fold dilution in fresh EZRDM and again grown to exponential phase (OD = 0.2–0.4). Cells were then plated on a poly-l-lysine–coated coverslip equipped with a CoverWell perfusion chamber (Electron Microscopy Sciences) of well volume 140 μl. Untreated cells grew normally under these conditions for at least 30 min. For the different drug treatments, when the bulk culture reached exponential phase, the cells were treated with the appropriate concentration of the drug and kept in the shaker for 30–40 min more. The drug concentrations were as follows: 20 μM Onc112 (20 × MIC), 300 μg/ml Cam (20 × MIC), and 5 mg/ml Ksg (30 × MIC). The cells were then plated as before. In all cases, the cells were imaged within ~5 min of plating.

**Imaging of E. coli cells**

Cells were imaged with an inverted microscope (model Eclipse-Ti, Nikon Instruments) equipped with an oil immersion objective (CFI Plan Apochromat Lambda DM 100× Oil, 1.45 NA), a 1.5× tube lens, and the Perfect Focus System. The localization and single-particle trajectory analyses were performed as described earlier (30, 38). For superresolution imaging, the individual fields of view were imaged for no longer than 20 s to minimize laser damage. Each prepared sample was imaged for no longer than 30 min. During this period untreated cells continued to grow normally.

For superresolution imaging of single copies of ribosomes (S2-mEos2 labeling) and EF-Tu (mEos2 labeling), the fluorescent protein mEos2 was activated using a 405-nm laser (CW laser, CrystaLaser); the photoconverted state was subsequently excited with a 561-nm laser (Sapphire CW laser, Coherent Inc., Bloomingfield, CT). Both lasers illuminated the sample for the entire duration of image acquisition. The 405-nm power density at the sample was 5–10 W/cm², which kept the average number of activated molecules in each camera frame to ~1. The 561-nm laser power density at the sample was ~8 kW/cm². Fluorescence was collected through a 610/75 bandpass filter (Chroma Technology Corp.). Sequential images were recorded by a back-plane–illuminated electron-multiplying charge-coupled device camera (iXon DV-860, Andor Technology USA, South Windsor, CT) at the rate of 485 Hz (2.06 ms/frame). The camera chip comprises 128 × 128 pixels, each 24 μm × 24 μm in real space. The effective pixel size after 150× magnification is 0.16 μm × 0.16 μm.

For widefield, dual-color imaging of S2-YFP and Sytox Orange–stained DNA in the same cell, the fluorescence images were recorded on a different electron-multiplying charge-coupled device camera (iXon DV-897, 512 × 512 pixels, each 16 μm × 16 μm; Andor Technology USA). The effective pixel size after 150× magnification is 0.106 μm × 0.106 μm. In the dual-color experiments, the program μManager (51) was used to obtain the data and to switch filters between frames using an LB10-NW filter wheel (Sutter Instrument). The movies comprised sequential green fluorescence (488-nm excitation), red fluorescence (561-nm excitation), and phase contrast images with 50-ms exposure time for each. To minimize spectral bleed-through in the two-color experiments, we utilized the narrower filters HQ510/20m for the green channel and HQ600/50m for the red channel. Laser intensities at the sample were typically ~5 W/cm² at 488 nm and ~2.5 W/cm² at 561 nm.

**Cell length measurements**

Tip-to-tip cell length was estimated from phase contrast images. For time-lapse movies of Onc112-treated cells (Fig. 2a), we used Fiji (ImageJ) software to quantify the change in cell length over time. The distribution of cell lengths under different growth conditions (Fig. 2b) was obtained from the program Oufi using the parameters optimized for E. coli as provided with the software (52). Results from Fiji and Oufi applied to the same image are similar.

**Single-molecule image analysis**

The fluorescence images were analyzed using a MATLAB graphical user interface developed in our laboratory as described previously (30, 31). Noise was attenuated by two different digital filters. Fluorescent signals were then identified using a peak-finding algorithm with a user-defined single-pixel...
intensity threshold. A particle is identified if the local intensity maximum is higher than the threshold. The threshold is carefully chosen so that the algorithm can distinguish between background and signal and avoid cutting trajectories unduly short.

Particles were located with sub-pixel resolution using a centroid algorithm. Rapidly moving molecules have images that are blurred asymmetrically due to diffusion during the camera frame. Centroid fitting can locate these particles with better accuracy than Gaussian fitting. The centroid algorithm is also faster computationally. A 7 × 7 pixel box was drawn around the intensity maximum, and the centroid of all the pixel intensities within the box was calculated. The centroid positions from successive frames were connected to form a trajectory only if the successive locations lay within 3 pixel = 480 nm of each other. A modified MATLAB version of the tracking program written by Crocker and Grier was applied to form the trajectories (53).

**Mean-square displacement plots MSD(τ)**

For free diffusion in an infinite space, the mean diffusion coefficient averaged over all molecules can be obtained from the MSD as a function of lag time τ. Here, $\text{MSD}(\tau) = \langle (r(t+\tau < r_m) + < \langle r_m > \tau) - r(t) \rangle^2 \rangle$, where $r(t)$ is the two-dimensional location of the particle at time $t$, $\tau$ is the lag time, and the average is taken over all times $t$ and over many trajectories (36). The slope of the first two points of an MSD(τ) plot provides an estimate of the mean diffusion coefficient: $D_{\text{Mean}} = \text{slope}/4$. The localization error $\sigma$ can be estimated from the $y$-intercept of the plot (36). For rapidly diffusing species such as free EF-Tu or free TCS imaged at 2 ms/frame, even six-step (12 ms) trajectories are significantly restricted by confinement. This contributes to downward curvature of the MSD plot. Hence the estimated mean diffusion coefficient yields a lower bound of the true $D_{\text{Mean}}$. To account for confinement, we used simulated numerical trajectories for analysis of single-step displacement distributions $P(r)$, as described next.

We calculated a 2D diffusion coefficient instead of a 3D one to ease the data analysis. The *E. coli* cell is symmetrical in the $y$- and $z$-axes. As the proteins undergo Brownian motion, their diffusion along both $y$ and $z$-axes should be similar. Thus, we still obtained their entire diffusive information in 2D. In the case a protein undergoes directed motion, a 2D diffusion analysis will wrongly estimate its diffusive property.

**Monte Carlo simulations of diffusive trajectories**

Even for a homogeneous sample (all particles having the same diffusion coefficient), there is no analytical formula for $P(r)$ that includes confinement effects and localization error. Instead, we simulated numerical diffusive trajectories using Monte Carlo methods in a spherocylindrical volume. As described previously (30, 31), we fit the experimental single-step displacement distribution $P(r)$ as a sum of two simulated distributions to account for confinement effects and localization error. We assumed that the two diffusive states have different localization errors, $\sigma_{\text{slow}}$ and $\sigma_{\text{fast}}$. The estimation of $\sigma_{\text{slow}}$ and $\sigma_{\text{fast}}$ is explained in detail in our previous papers (30, 31). Then we simulated a large number of random walk trajectories, each moving inside the spherocylinder with a particular diffusion coefficient $D$ and with localization error $\sigma_{\text{slow}}$ or $\sigma_{\text{fast}}$. Each set of simulations was carried out in a confining spherocylinder, which mimics the average dimension of an *E. coli* cell for a specific growth condition and with a fixed $D$ and $\sigma$ and camera exposure time of 2 ms. For each parameter set, we calculated 5000 six-step trajectories with molecules initially located randomly within the spherocylinder. The sub-trajectory of each molecule during each camera frame was computed for 1000 microsteps of 2–μs duration. The centroid of each resulting 2-ms sub-trajectory was computed; localization error was included by sampling a Gaussian of appropriate $\sigma$. Trajectories were formed by joining the resulting locations over time. The 5000 trajectories were then used to compute a numerical single-step probability distribution $P_{\text{model}}(r|D)$ for use in the least-squares analysis of the corresponding experimental distribution.

**Fitting of single-step $P(r)$ distributions to a static, two-state model**

For every condition studied, experimental trajectories that lasted at least six steps were selected for analysis; longer trajectories were truncated after the sixth step. Each experimental single-step displacement was calculated as $r_i = \sqrt{(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2}$; these were pooled from all trajectories to form the experimental distribution $P(r)$. We fit the experimental distribution $P(r)$ to a weighted average of two static populations, in a least-squares sense. For two states, the fitting function is $P_{\text{model}}(r|D) = f_{\text{slow}}P(r|D_{\text{slow}}) + (1 - f_{\text{slow}})P(r|D_{\text{fast}})$. Here the three fitting parameters are $D_{\text{fast}}$, $D_{\text{slow}}$, and the fractional population $f_{\text{slow}}$ which in turn fixes $f_{\text{fast}} = 1 - f_{\text{slow}}$. For all our fitting procedures, $D_{\text{slow}}$ was sampled from 0.05 to 3 μm$^2$/s with intervals of 0.05 μm$^2$/s and $D_{\text{fast}}$ was sampled from 0.1 to 9 μm$^2$/s with intervals of 0.1 μm$^2$/s. The goodness of each fit was evaluated by calculating the reduced chi-square statistic ($\chi^2_r$). We generated a 3D matrix of $\chi^2_r$ values, with each axis representing one of the fitting parameters. The parameters that gave the minimum $\chi^2_r$ were chosen as the best-fit. The $P(r)$ fitting and the error estimation for the fitting parameters are explained in further detail in our previous papers (30, 31).

**Data availability**

All raw data will be available on request. Please contact Dr. Mainak Mustafi (mm5701@cumc.columbia.edu).

**Acknowledgment**—We thank Dr. Somenath Bakshi for helpful scientific discussions.

**Author contributions**—Y. Z., J. C. W., and M. M. conceptualization; Y. Z. and M. M. data curation; Y. Z. and M. M. formal analysis; Y. Z., J. C. W., and M. M. writing—original draft; Y. Z., J. C. W., and M. M. writing—review and editing; J. C. W. resources; J. C. W. software; J. C. W. and M. M. supervision; J. C. W. funding acquisition;
Onc112 effects on translation machinery in live E. coli

J. C. W. and M. M. validation; J. C. W. and M. M. visualization; J. C. W. and M. M. project administration; M. M. investigation.

Funding and additional information—This work was supported by the National Institutes of Health Grant GM094510 (to J. C. W.) and by the National Science Foundation Grant MCB-1512946 (to J. C. W.). This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: EF-Tu, elongation factor-Tu; aa-tRNA, aminoacyl-tRNA; TC, ternary complex; EZRDM, EZ rich defined medium; MIC, minimum inhibitory concentration; Onc112, oncocin112; Cam, chloramphenicol; Ksg, kasugamycin; PrAMP, proline-rich antimicrobial peptide; aa, amino acid; AMP, antimicrobial peptide; MSD, mean-square displacement.

References

1. Kohanski, M. A., Dwyer, D. J., and Collins, J. J. (2010) How antibiotics kill bacteria: from targets to networks. Nat. Rev. Microbiol. 8, 423–435 CrossRef Medline
2. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3, 238–250 CrossRef Medline
3. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature 415, 389–395 CrossRef Medline
4. (2019) Antimicrobial peptides: basics for clinical application (Matsuzaki, K. ed.), Springer, Singapore
5. Guilhelmelli, F., Vilela, N., Albuquerque, P., Derengowski, Lda S., Silva-Pereira, I., and Kyaw, C. M. (2013) Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. Front. Microbiol. 4, 1–12 CrossRef Medline
6. Zhu, Y., Mohapatra, S., and Weisshaar, J. C. (2019) Rigidification of the Escherichia coli cytoplasm by the human antimicrobial peptide LL-37 revealed by superresolution fluorescence microscopy. Proc. Natl. Acad. Sci. U. S. A. 116, 1017–1026 CrossRef Medline
7. Chongsrirawatanawat, N. P., Lin, J. S., Kapoor, R., Wetzler, M., Rea, J. C. A., Didwania, M. K., Contag, C. H., and Barron, A. E. (2017) Intracellular biomass flocculation as a key mechanism of rapid bacterial killing by cationic, amphipathic antimicrobial peptides and peptoids. Sci. Rep. 7, 16718 CrossRef Medline
8. Casteels, P., and Tempst, P. (1994) Apidaecin-type peptide antibiotics function through a non-pore-forming mechanism involving stereospecificity. Biochem. Biophys. Res. Commun. 199, 339–345 CrossRef Medline
9. Graf, M., Mardirossian, M., Nguyen, F., Seefeldt, A. C., Guichard, G., Scocchi, M., Innis, C. A., and Wilson, D. N. (2017) Proline-rich antimicrobial peptides targeting protein synthesis. Nat. Prod. Rep. 34, 702–711 CrossRef Medline
10. Polikanov, Y. S., Aleksashin, N. A., Beckert, B., and Wilson, D. N. (2018) The mechanisms of action of ribosome-targeting peptide antibiotics. Front. Mol. Biosci. 5, 1–21 CrossRef Medline
11. Graf, M., and Wilson, D. N. (2019) Intracellular antimicrobial peptides targeting the protein synthesis machinery. Adv. Exp. Med. Biol. 1117, 73–89 CrossRef Medline
12. Mattiuzzo, M., Bandiera, A., Gennaro, R., Benincasa, M., Pacor, S., Antcheva, N., and Scocchi, M. (2007) Role of the Escherichia coli SbmA in the antimicrobial activity of proline-rich peptides. Mol. Microbiol. 66, 151–163 CrossRef Medline
13. Seefeldt, A. C., Graf, M., Pérébashine, N., Nguyen, F., Arenz, S., Mardirossian, M., Scocchi, M., Wilson, D. N., and Innis, C. A. (2016) Structure of the mammalian antimicrobial peptide Bac7(1–16) bound within the exit tunnel of a bacterial ribosome. Nucleic Acids Res. 44, 2429–2438 CrossRef Medline
14. Mardirossian, M., Pérébashine, N., Benincasa, M., Gambato, S., Hofmann, S., Huter, P., Muller, C., Hilpert, K., Innis, C. A., Tossi, A., and Wilson, D. N. (2018) The dolphin proline-rich antimicrobial peptide Tur1A inhibits its protein synthesis by targeting the bacterial ribosome. Cell Chem. Biol. 25, 530–539.e7 CrossRef Medline
15. Knappe, D., Piantavigna, S., Hansen, A., Mechler, A., Binas, A., Nolte, O., Martin, L. M., and Hoffmann, R. (2010) Oncocin (VDKPYLPFRPRPRPR-RYINR-NH3): a novel antibacterial peptide optimized against gram-negative human pathogens. J. Med. Chem. 53, 5240–5247 CrossRef Medline
16. Knappe, D., Kabankov, N., and Hoffmann, R. (2011) Bactericidal oncocin derivatives with superior serum stabilities. Int. J. Antimicrob. Agents 37, 166–170 CrossRef Medline
17. Roy, R. N., Lomakin, I. B., Gagnon, M. G., and Steitz, T. A. (2015) The mechanism of inhibition of protein synthesis by the proline-rich peptide oncocin. Nat. Struct. Mol. Biol. 22, 466–469 CrossRef Medline
18. Seefeldt, A. C., Nguyen, F., Antunes, S., Pérébashine, N., Graf, M., Arenz, S., Inampudi, K. K., Douat, C., Guichard, G., Wilson, D. N., and Innis, C. A. (2015) The proline-rich antimicrobial peptide Onc112 inhibits translation by blocking and destabilizing the initiation complex. Nat. Struct. Mol. Biol. 22, 470–475 CrossRef Medline
19. Weaver, J., Mohammad, F., Buskirk, A. R., and Storz, G. (2019) Identifying small proteins by ribosome profiling with stalled initiation complexes. mBio 10, e02819-18 CrossRef Medline
20. Bulky, D., Innis, C. A., Blaha, G., and Steitz, T. A. (2010) Revisiting the structures of several antibiotics bound to the bacterial ribosome. Proc. Natl. Acad. Sci. U. S. A. 107, 17158–17163 CrossRef Medline
21. Baksí, S., Choi, H., Mondal, J., and Weisshaar, J. C. (2014) Time-dependent effects of transcription- and translation-halting drugs on the spatial distributions of the E. coli chromosome and ribosomes. Mol. Microbiol. 94, 871–887 CrossRef Medline
22. Schluenzen, F., Takemoto, C., Wilson, D. N., Kaminishi, T., Harms, J. M., Hanawa-Suetsugu, K., Szafarski, W., Kawazoe, M., Shirouzu, M., Shirouzu, M., Nierhaus, K. H., Yokoyama, S., and Fucini, P. (2006) The antibiotic kasugamycin mimics mRNA nucleotides to destabilize tRNA binding and inhibit canonical translation initiation. Nat. Struct. Mol. Biol. 13, 871–878 CrossRef Medline
23. Schuwirth, B. S., Day, J. M., Hau, C. W., Janssen, G. R., Dahlberg, A. E., Cate, J. H., and Vila-Sanjurjo, A. (2006) Structural analysis of kasugamycin inhibition of translation. Nat. Struct. Mol. Biol. 13, 879–886 CrossRef Medline
24. Agrawal, A., and Weisshaar, J. C. (2018) Effects of alterations of the E. coli lipopolysaccharide layer on membrane permeabilization events induced by Cecropin A. Biochim. Biophys. Acta. Biomembr. 1860, 1470–1479 CrossRef Medline
25. Choi, H., Kang, H., and Weisshaar, J. C. (2016) Lights, camera, action! Antimicrobial peptide mechanisms imaged in space and time. Trends Microbiol. 24, 111–122 CrossRef Medline
26. Sochacki, K. A., Bars, K. J., Bucki, R., and Weisshaar, J. C. (2011) Real-time attack on single Escherichia coli cells by the human antimicrobial peptide LL-37. Proc. Natl. Acad. Sci. U. S. A. 108, E77–E81 CrossRef Medline
27. Choi, H., Yang, Z., and Weisshaar, J. C. (2015) Single-cell, real-time detection of oxidative stress induced in Escherichia coli by the antimicrobial peptide CM15. Proc. Natl. Acad. Sci. U. S. A. 112, E303–E310 CrossRef Medline
28. Baksí, S., Choi, H., and Weisshaar, J. C. (2015) The spatial biology of transcription and translation in rapidly growing Escherichia coli. Front. Microbiol. 6, 363 CrossRef Medline
29. Zhu, Y., Mustafi, M., and Weisshaar, J. C. (2020) Biophysical properties of Escherichia coli cytoplasm in stationary phase probed by superresolution fluorescence microscopy. mBio 11, e00143 CrossRef Medline
30. Mustafi, M., and Weisshaar, J. C. (2018) Simultaneous binding of multiple EF-Tu copies to translating ribosomes in live Escherichia coli. mBio 9, e02143 CrossRef
Onc112 effects on translation machinery in live E. coli

31. Mustafi, M., and Weisshaar, J. C. (2019) Near saturation of ribosomal L7/L12 binding sites with ternary complexes in slowly growing E. coli. J. Mol. Biol. 431, 2343–2353 CrossRef Medline
32. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., et al. (2012) Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 CrossRef Medline
33. Scocchi, M., Tossi, A., and Gennaro, R. (2011) Proline-rich antimicrobial peptides: converging to a non-lytic mechanism of action. Cell. Mol. Life Sci. 68, 2317–2330 CrossRef Medline
34. Bakshi, S., Siryporn, A., Goulian, M., and Weisshaar, J. C. (2012) Superresolution imaging of ribosomes and RNA polymerase in live Escherichia coli cells. Mol. Microbiol. 85, 21–38 CrossRef Medline
35. Berg, H. C. (1993) Random Walks in Biology, Princeton University Press.
36. Michalet, X. (2010) Mean square displacement analysis of single-particle trajectories with localization error: Brownian motion in isotropic medium. Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 82, 041914 CrossRef Medline
37. Gray, W. T., Govers, S. K., Xiang, Y., Parry, B. R., Campos, M., Kim, S., and Babitzke, P., and O.
38. Diaconu, M., Kothe, U., Schlunzen, F., Fischer, N., Harms, J. M., Tonevitsky, A. G., Stark, H., Rodnina, M. V., and Wahl, M. C. (2005) Structural basis for the function of the ribosomal L7/L12 stalk in factor binding and GTPase activation. Cell 121, 991–1004 CrossRef Medline
39. Kothe, U., Wieden, H. J., Mohr, D., and Rodnina, M. V. (2004) Interaction of helix D of elongation factor Tu with helices 4 and 5 of protein L7/L12 on the ribosome. J. Mol. Biol. 336, 1011–1021 CrossRef Medline
40. Traut, R. R., Dey, D., Bochkariyov, D. E., Oleinikov, A. V., Jokhadze, G. G., Hamman, B., and Jameson, D. (1995) Location and domain structure of Escherichia coli ribosomal protein L7/L12: site specific cysteine crosslinking and attachment of fluorescent probes. Biochem. Cell Biol. 73, 949–958 CrossRef Medline
41. Dennis, P. P., and Bremer, H. (1974) Differential rate of ribosomal protein synthesis in Escherichia coli B/r. J. Mol. Biol. 84, 407–422 CrossRef Medline
42. Kothe, U., Wieden, H. J., Mohr, D., and Rodnina, M. V. (2004) Interaction of translation across bacteria. Cell 113, 1017–1026 CrossRef Medline
43. Jacobs-Wagner, C. (2019) Nucleoid size scaling and intracellular organization and attachment of fluorescent probes. Biochem. Cell Biol. 97, 494–508 CrossRef Medline
44. Mustafi, M., and Weisshaar, J. C. (2019) Near saturation of ribosomal L7/L12 binding sites with ternary complexes in slowly growing E. coli. J. Mol. Biol. 431, 2343–2353 CrossRef Medline
45. Rudorf, S., Thommen, M., Rodnina, M. V., and Lipowsky, R. (2014) Deducing the kinetics of protein synthesis in vivo from the transition rates measured in vitro. PLoS Comput. Biol. 10, e1003909 CrossRef Medline
46. Rudorf, S., and Lipowsky, R. (2015) Protein synthesis in E. coli: dependence of codon-specific elongation on tRNA concentration and codon usage. PLoS ONE 10, e0134994 CrossRef Medline
47. Wohlgemuth, I., Pohl, C., Mittelstaedt, J., Konevega, A. L., and Rodnina, M. V. (2011) Evolutionary optimization of speed and accuracy of decoding on the ribosome. Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 2979–2986 CrossRef Medline
48. Morse, J. C., Girodat, D., Burnett, B. J., Holm, M., Altman, R. B., Sanbonmatsu, K. Y., Wieden, H.-J., and Blanchard, S. C. (2020) Elongation factor-Tu can repetitively engage aminoacyl-tRNA within the ribosome during the proofreading stage of tRNA selection. Proc. Natl. Acad. Sci. U. S. A. 117, 3610–3620 CrossRef Medline
49. Thiel, A., Valens, M., Vallet-Gely, I., Espeli, O., and Boccard, F. (2012) Long-range chromosome organization in E. coli: a site-specific system isolates the Ter macrodomain. PLoS Genet. 8, e1002672 CrossRef Medline
50. Edelstein, A. D., Tsuchida, M. A., Amodaj, N., Pinkard, H., Vale, R. D., and Stuurman, N. (2014) Advanced methods of microscope control using μManager software. J. Biol. Methods 1, e10 CrossRef Medline
51. Paintdakhi, A., Parry, B., Campos, M., Irnov, I., Elf, J., Surovtsev, I., and Jacobs-Wagner, C. (2016) Oufti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. Mol. Microbiol. 99, 767–777 CrossRef Medline
52. Crocker, J. C., and Grier, D. G. (1996) Methods of digital video microscopy for colloidal studies. J. Colloid Interface Sci. 179, 298–310 CrossRef Medline