

Context-specific inhibition of translation by ribosomal antibiotics targeting the peptidyl transferase center

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The first broad-spectrum antibiotic chloramphenicol and one of the newest clinically important antibacterials, linezolid, inhibit protein synthesis by targeting the peptidyl transferase center of the bacterial ribosome. Because antibiotic binding should prevent the placement of aminoacyl-tRNA in the catalytic site, it is commonly assumed that these drugs are universal inhibitors of peptidyl transfer and should readily block the formation of every peptide bond. However, our *in vitro* experiments showed that chloramphenicol and linezolid stall ribosomes at specific mRNA locations. Treatment of bacterial cells with high concentrations of these antibiotics leads to preferential arrest of translation at defined sites, resulting in redistribution of the ribosomes on mRNA. Antibiotic-mediated inhibition of protein synthesis is most efficient when the nascent peptide in the ribosome carries an alanine residue and, to a lesser extent, serine or threonine in its penultimate position. In contrast, the inhibitory action of the drugs is counteracted by glycine when it is either at the nascent-chain C terminus or at the incoming aminoacyl-tRNA. The context-specific action of chloramphenicol illuminates the operation of the mechanism of inducible resistance that relies on programmed drug-induced translation arrest. In addition, our findings expose the functional interplay between the nascent chain and the peptidyl transferase center.

ribosome | antibiotics | protein synthesis | nascent peptide | oxazolidinones

The key chemical reaction catalyzed by the ribosome is peptide bond formation. This reaction occurs in the peptidyl transferase center (PTC) where the transfer of the nascent peptide from the P-site-bound peptidyl-tRNA (pept-tRNA) to the A-site-bound aminoacyl tRNA (aa-tRNA) leads to elongation of the growing protein chain by one amino acid at each round of translation (reviewed in ref. 1). Although the peptidyl transfer reaction is potentially assisted by the chemical environment of the PTC (2–4), the main contribution of the ribosome to peptide bond formation is entropic, as the major acceleration of the reaction stems from the proper orientation of the donor and acceptor substrates within the catalytic center (5–7).

The PTC is a primary target for antibiotics that inhibit protein synthesis (see ref. 8 for review), such as chloramphenicol (CHL), one of the first broad-spectrum antibacterials in clinical use (9). CHL binds to the PTC A site, preventing proper placement of the aminoacyl moiety of aa-tRNA (Fig. 1) (10–12). Because the key interactions with the ribosome in the A site are shared by all aa-tRNAs, CHL is viewed as a universal inhibitor of peptide bond formation (reviewed in ref. 13). However, this commonly accepted model of CHL action fails to explain several experimental observations such as the differential inhibition of translation of specific mRNA templates (14, 15) or only partial inhibition of puromycin-mediated release of nascent chains in polysomes even at saturating CHL concentrations (16). The perceived ability of CHL to indiscriminately inhibit formation of any peptide bond also conflicts with its role as an inducer of resistance genes. Activation of a number of CHL resistance genes relies on the antibiotic-promoted arrest of translation at a specific codon within the regulatory leader ORF (reviewed in ref. 17). Thus, to activate the expression of the resistance locus in response to the antibiotic assault, the ribosome should be able to progress through several leader ORF codons to reach the site of the programmed

translation arrest. How the ribosome can polymerize a segment of the leader peptide, if CHL indiscriminately inhibits formation of any peptide bond, remained unclear.

Although CHL is one of the oldest known antibiotics, linezolid (LZD) belongs to one of the newest classes of clinically important PTC-targeting protein synthesis inhibitors, the oxazolidinones (Fig. 1). Genetic, biochemical, and crystallographic evidence showed that LZD binds in the PTC A site, at a location overlapping with the CHL binding pocket. Similar to CHL, LZD directly clashes with the placement of the aminoacyl moiety of the aa-tRNA, suggesting that the drug should indiscriminately inhibit peptidyl transfer reaction (Fig. 1) (18–21). It remained unexplained, however, why LZD, which readily interfered with *in vivo* protein synthesis or cell-free translation (22), failed to inhibit peptide bond formation between fMet-tRNA and puromycin (18, 23).

Because of the accumulating biochemical evidence that contradicted the conventional view of the mode of action of CHL and LZD, we questioned whether these antibiotics universally block the formation of every peptide bond during protein synthesis. Here, we show that CHL and LZD do not act as global inhibitors of peptide bond formation, but rather block translation at specific locations within the mRNA in a context-specific manner. Furthermore, we

Significance

Chloramphenicol and linezolid interfere with translation by targeting the ribosomal catalytic center and are viewed as universal inhibitors of peptide bond formation. We show that, contrary to this view, the activity of these antibiotics critically depends on the nature of specific amino acids of the nascent chain carried by the ribosome and by the identity of the residue entering the A site. These findings indicate that the nascent protein modulates the properties of the ribosomal catalytic center and affects binding of its ligands. Understanding the principles of context specificity of ribosomal drugs may help develop better antibiotics.

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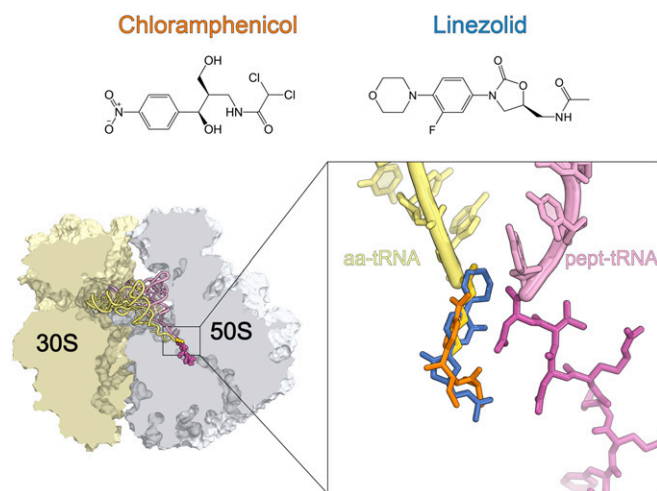


Fig. 1. The binding site of CHL and LZD in the peptidyl transferase center of the 50S ribosomal subunit. (Top) The chemical structures of CHL and LZD. (Bottom) When bound to the large ribosomal subunit in the PTC A site, the molecules of CHL (orange) and LZD (blue) would sterically clash with the aminoacyl moiety of the aminoacyl-tRNA (yellow). P-site-bound peptidyl-tRNA esterified with the nascent peptide chain is purple. The images were prepared based on the structures with the Protein Data Bank ID codes 4V77, 3DLL, and 3J5L (10, 20, 46).

present evidence that the specificity of action of these drugs is defined by the nature of the penultimate residue of the nascent peptide as well as by the amino acid residues directly participating in peptide bond formation. Our findings unveiled an important functional influence of the nascent peptide on the properties of the PTC and expand the concept of the context-specific action of ribosomal antibiotics.

Results

Toeprinting Analysis Reveals Site Specificity of CHL and LZD Action.

To assess the mode of action of CHL and LZD in vitro, we used toeprinting analysis, which determines the position of the antibiotic-stalled ribosome on an mRNA (24, 25). If, as alleged, CHL and LZD act as global inhibitors of the PTC catalytic function, preincubation of the ribosome with these antibiotics should prevent the formation of the very first peptide bond. However, consistent with our previous observations (25), even at very high concentrations of CHL or LZD, ribosomes were not arrested at the initiator codon of any of the tested mRNA templates (Fig. S14). Instead, translation progressed through few codons until the ribosome was stalled, with varying efficiency, at specific downstream sites. In an attempt to understand the requirement for the preferential action of CHL and LZD at specific mRNA locations, we aligned the encoded protein sequences leading up to the experimentally identified sites of CHL- or LZD-induced arrest and examined the nature of the P- or A-site amino acids directly participating in peptide bond formation (Fig. S1B). Nonetheless, no clear conservation of the donor or acceptor residues was immediately obvious. We realized that, to search for the motifs conducive to the action of CHL and LZD, we needed to operate with a broader array of sites of the preferred action of these drugs. Therefore, we used ribosome profiling, which allows analyzing the mode of action of protein synthesis inhibitors at the genome-wide level (26–29).

Identity of the Penultimate Residue of the Nascent Peptide Is Critical for the Action of CHL and LZD. The ribosome-profiling experiments were carried out with the *Escherichia coli* strain BWDK, a descendant of the WT *E. coli* K-12 strain, where the absence of the *tolC* gene (a key component of the multidrug efflux pump) renders the cells hypersusceptible to antibiotics. Exponentially growing cells were exposed to a 100-fold excess over the minimal inhibitory concentration of CHL or LZD for 2.5 min, a time period sufficient to reach maximum

inhibition of translation (Fig. S2). The ribosome-protected mRNA fragments were then prepared, sequenced, and mapped to the genome using established procedures (30, 31). Treatment with any of the two inhibitors caused a modest redistribution of ribosome density along the genes relative to the untreated control (Fig. S3). Thus, it became evident that exposure to the antibiotic does not immediately “freeze” translation. Instead, ribosomes can still polymerize a few peptide bonds before pausing at particular codons. This observation is consistent with our in vitro toeprinting results, which showed that CHL and LZD stall translation at a number of specific locations within the protein-coding sequences (Fig. S1).

We identified the preferential sites of antibiotic action by computing changes in ribosome occupancy at ~60,000 individual codons between the antibiotic-treated and untreated cells and ranking all of the analyzed codons by the magnitude of the change (Fig. 2) (see *SI Materials and Methods* for detail). For each antibiotic, we then selected the top 1,000 codons, where the strongest drug-induced translation arrest was observed. Within these sites, we searched for a specific sequence signature among amino acids encoded within the nine codons preceding the arrest site (positions –1 to –9), the arrest codon (position 0), which occupies the P site of the stalled ribosome, and the following codon (position +1), corresponding to the A-site codon (Fig. 2). Remarkably, the preferential CHL arrest sites showed significant enrichment in Ala (38.1%) and, to a lesser extent, of Ser (14.8%) or Thr (6.3%) codons, in the –1 position compared with the expected random occurrence of these residues (15.2%, 7.8%, and 5.5%, respectively) (Fig. 2A and Fig. S4). The sites of LZD-induced arrest exhibited an even stronger preference for Ala codons (69.9%) in the same position (Fig. 2B and Fig. S4). Although Ala and Thr can be defined by four codons each and Ser is defined by six codons, no preference for any specific Ala, Ser, or Thr codon at the sites of arrest was apparent. This lack of codon bias argues that the specificity of antibiotic action is defined by the nature of the encoded amino acids rather than the mRNA sequence or tRNA structure. The occurrence of Ala, Ser, or Thr in the penultimate peptide position strongly correlated with the drug-induced translation stalling throughout the entire range of the analyzed locations, and their presence progressively decreased toward the end of the spectrum where codons with the least pronounced ribosome stalling were grouped (Fig. S5A). Furthermore, when all of the ~60,000 analyzed sites were grouped into 20 bins, according to the nature of the nascent-chain penultimate residue, the sites with Ala, Ser, or Thr in position (–1) showed a significant increase of the cumulative ribosome occupancy across all of the analyzed codons (Fig. S5B). All of these data strongly argue that presence of Ala (and, to some degree, Thr or Ser) as the penultimate amino acid in the nascent chain stimulates the inhibitory action of CHL and LZD.

To further examine the influence of the sequence context on drug-induced arrest, we selected a well-defined model system appealingly suited for cell-free translation experiments (32). According to the ribosome-profiling data, the drug-induced ribosome stalling at the Leu5 codon of the *hns* gene represents one of the 10 strongest arrest sites common for both CHL and LZD (Fig. 3A). Drug-induced ribosome stalling at the *hns* Leu5 codon was readily reproduced in vitro in the toeprinting assay (Fig. 3B). By comparing the fraction of the ribosomes arrested at the Leu5 codon with those that bypassed the site of drug-induced translation stalling, we compared the efficiency of translation inhibition for the H-NS mutants containing every possible amino acid substitutions of Ala4 (Fig. 3C and D). The results of the in vitro experiments confirmed that the presence of Ala in the penultimate position of the H-NS N-terminal pentapeptide significantly enhanced the action of CHL and LZD. Replacing Ala4 with any other amino acid greatly reduced the efficiency of drug-induced ribosome stalling. Furthermore, in agreement with the profiling data (Fig. S4), Ser and, in the case of LZD, also Thr at position –1 were more conducive to the antibiotic action than any other substitution of Ala4 (Fig. 3C and D).

Having verified the importance of the penultimate peptide residue in the mechanism of LZD and CHL action, we reexamined the collection of the arrest sites identified by toeprinting in our initial experiments (Fig. S1B). Consistent with the trend observed in vivo,

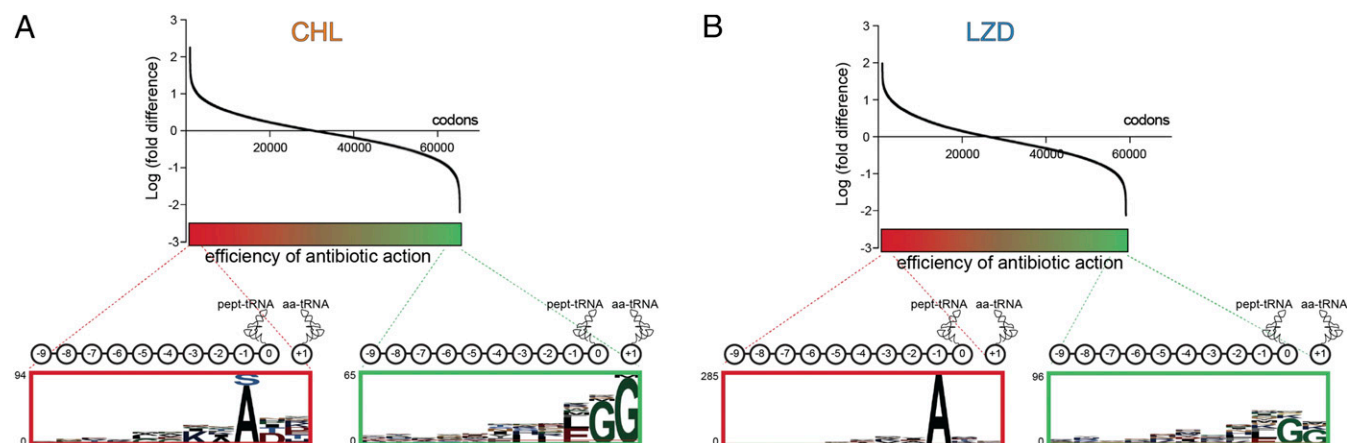


Fig. 2. Context specificity of the action of CHL and LZD in vivo. (A and B) Ranking of genomic sites according to the relative fold difference in the ribosome occupancy in cells treated with CHL (A) or LZD (B) vs. the untreated control. The higher relative fold difference values (left side of the spectrum, colored in red) correspond to the sites of more pronounced inhibition of translation by the antibiotics. The low fold difference values (right side of the spectrum, shown in green) represent the sites where antibiotics were least efficient. The magnified panels at the *Bottom* show the pLogo analysis of amino acid bias within the top 1,000 (red frame) and bottom 1,000 (green frame) sites of action of CHL or LZD. The 10 C-terminal residues of the nascent chains (marked as 0 to –9 of pept-tRNA) and the incoming amino acids (marked as +1, aa-tRNA) in these sites are indicated in the cartoons. The y axes of the pLogo panels correspond to the odds of the binomial probability (in logarithmic scale) of occurrence of specific amino acids at the defined positions (47).

more than 80% of the drug-induced arrests detected in those experiments occurred when the penultimate amino acid of the nascent peptide was Ala, Ser, or Thr (marked by arrowheads in Fig. S1B).

Collectively, the results of the genome-wide analysis and of the in vitro testing converge on the key stimulatory role of Ala, Ser, or Thr in the penultimate position of the nascent peptide in the mechanism of action of the PTC-targeting antibiotics CHL and LZD.

CHL and LZD Poorly Inhibit Peptide Bond Formation When Glycine Residues Are Involved. The codons with the maximally increased ribosomal occupancy in drug-treated cells represent the sites where antibiotic action is most pronounced (red frames in Fig. 2). Conversely, the codons whose occupancy significantly decreases in the presence of the drug are those where the antibiotic fails to hinder peptide bond formation. Despite the presence of the inhibitor, the ribosomes proceed with translation, traversing these codons until they encounter the nearest “strong” arrest location. In contrast to our finding for the top arrest sites, the analysis of the 1,000 codons characterized by the most dramatic decrease in ribosome occupancy in the drug-treated cells (green frames in Fig. 2) did not show a dramatic bias in favor of any particular amino acid in the position (–1) of the peptide. Instead, it revealed a strong prevalence for the presence of glycine residues in either the P site (position 0) or the A site (position +1) of the translating ribosome. These data suggest that the peptidyl transfer reaction involving a Gly residue either as a donor or as an acceptor is poorly inhibited by CHL or LZD.

The occurrence of Gly codons in either the P or A sites negatively correlated with the efficiency of drug-inflicted stalling throughout the entire range of analyzed sites (Fig. S5A). Consistently, the presence of glycine codons in the P or A sites correlated with the most pronounced decrease of the cumulative ribosomal density at the corresponding codons in antibiotic-treated cells (Fig. S5B). These findings derived from the ribosome-profiling analysis were in keeping with the results of the subsequent in vitro testing with the *hns* gene: presence of a Gly residue in the P or the A site made the action of CHL or LZD inefficient (Fig. 3 D and E).

Thus, we concluded that the amino acid sequence context is capable of either stimulating or counteracting the inhibition of peptide bond formation by CHL and LZD.

Induction of CHL Resistance Genes Relies on the Stimulatory Effect of the Penultimate Residue of the Nascent Chain on Drug Action. Our newly gained understanding of the site specificity of CHL action

prompted us to reevaluate the mechanism of inducible CHL resistance. We analyzed CHL-dependent programmed translation arrest at the leader ORFs of two resistance genes, *catA86* (originated in Gram-positive bacteria) and *cmlA* (common to Gram-negative species) (33) (Fig. 4A). Toeprinting analysis showed that, in the presence of the antibiotic, translation driven by *E. coli* ribosomes stalls when the fifth codon of *catA86L* or the eighth codon of the *cmlA* ORF enter the ribosomal P site (Fig. 4 B and C). The same stalling site was observed when translation of *cat86AL* was catalyzed by ribosomes isolated from Gram-positive *Bacillus subtilis* (Fig. S6A). These results are in line with the conclusions of the previous genetic testing, which suggested that the N-terminal pentapeptide MVKTD of Cat86AL or the octapeptide MSTSKNAD of CmlAL are synthesized in the presence of CHL (33, 34). Remarkably, translation arrest at the leader ORFs occurs when the nascent chain carries in the penultimate positions amino acid residues conducive to CHL action: Ala (in the case of CmlAL) or Thr (Cat86AL) (Fig. 4 B and C). Thus, the mechanism of induction of CHL resistance simply exploits the site specificity rules that have emerged from the profiling and toeprinting analyses. In agreement with this assertion, in the alanine-scanning mutagenesis experiment carried out with *cat86AL* and *cmlA*, we observed the appearance of new ribosome stalling sites consistently occurring at the codon subsequent to the engineered Ala mutation (asterisks in Fig. 4 D and E). In addition, replacement of Thr4 of the wt Cat86AL with Ala significantly enhanced CHL-mediated stalling at the “native site” (compare lanes MVKTDK and MVKADK in Fig. 4D), corroborating our conclusion that an Ala residue in the penultimate peptide position is the most stimulatory for drug activity.

Codons 2–4 of the *cmlA* ORF specify Ser and Thr, which are expected to moderately stimulate CHL action (Fig. 3 and Fig. S4). Curiously, however, translation of the ORF in the presence of the antibiotic proceeds fairly efficiently until the eighth codon (large black triangle in Fig. 4C), with only a moderate arrest at the codon 5 (small black triangle in Fig. 4C). One possible explanation is that efficient CHL-induced stalling requires the nascent chain to reach a certain length. Indeed, sequential 5′-terminal truncations of the *cmlA* or *cat86AL* ORFs diminished the efficiency of drug-induced arrest at the native site when the nascent chain became shorter than 4 (Cat86AL) or 5 (CmlAL) aa long (Fig. 4 F and G).

Although the regulatory ORFs of the CHL resistance genes have been evolutionarily selected to respond specifically to the presence of natural CHL, the similarity in the context specificity of action of CHL and LZD suggested that the latter, a synthetic antibiotic, could

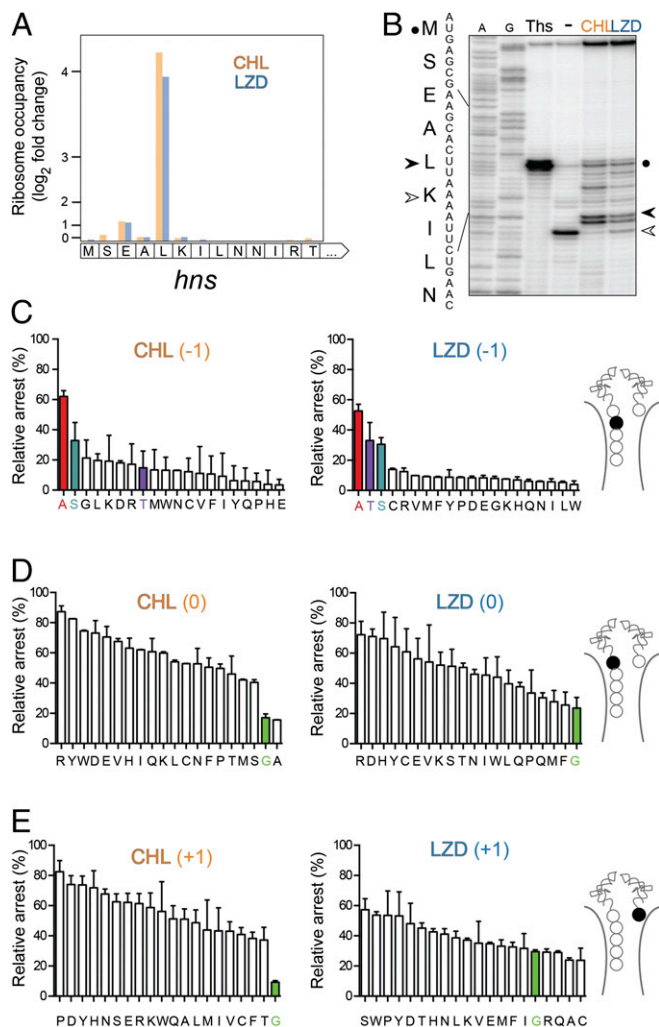


Fig. 3. Amino acid residues of the PTC donor and acceptor substrates influence antibiotic action. (A) Changes in the ribosome occupancy of the first codons of the *hns* gene in cells treated with CHL or LZD compared with that in the untreated cell culture. (B) In vitro toeprinting analysis of CHL- or LZD-induced ribosome stalling close to the 5'-end of the *hns* gene. The control antibiotic thiostrepton (Ths) was used to arrest translation at the start codon (black circle). The prominent CHL- and LZD-induced arrest site at the Leu5 codon of the gene is indicated by a black arrowhead. Due to the presence of the Ile-tRNA synthetase inhibitor mupirocin in all of the samples, the ribosomes that were not arrested by CHL or LZD at the *hns* Leu5 codon, were "caught" at the following Lys6 codon (open arrowhead). A- and G-specific sequencing lanes are indicated. The sequence of the first nine codons of the *hns* gene and the encoded amino acids are indicated on the side of the gel. (C–E) The effect of mutagenizing (C) the Ala4 (position –1), (D) Leu5 (position 0), or (E) Lys6 (position +1) codons of *hns* on CHL- or LZD-induced translation arrest. The cartoons showing the PTC region of the CHL- or LZD-stalled ribosomal complexes highlight the mutagenized amino acid residue (filled spheres) in each set. The efficiency of antibiotic-mediated arrest ("relative arrest") was calculated by comparing the fraction of arrested ribosomes at codon 5 (estimated from the intensity of the CHL- or LZD-specific toeprint bands) with the fraction of ribosomes trapped at codon 6 (calculated from the intensity of the mupirocin-specific toeprint bands) during the translation of the WT or mutant *hns* templates (see B for reference). For the templates where the specified codon was replaced with an Ile codon, the Ile7 codon was mutated to Thr and borrelidin (and inhibitor of Thr-RS) was used instead of mupirocin. The bars representing H-NS mutants with Ala, Ser, or Thr in the penultimate peptide position are highlighted in red; those corresponding to the mutants with Gly in the P or A sites are highlighted in green. The error bars show deviation from the mean in two independent experiments.

also act as an inducer. Indeed, LZD and even the newest oxazolidinone antibiotic, tedizolid, were able to stimulate ribosome stalling at the fifth codon of *cat86AL* or the eighth codon of *cmlAL*, although less efficiently than CHL (Fig. S6). Thus, synthetic oxazolidinones likely would be able to induce the natural CHL resistance genes.

Discussion

We have presented evidence that antibiotics CHL and LZD, belonging to two different classes of ribosomal PTC inhibitors, do not actively block formation of every peptide bond, but instead interfere with translation in a context-specific manner. The nature of the two C-terminal nascent peptide residues as well as of the A-site acceptor strongly influence the ability of these drugs to inhibit peptidyl transfer. The presence of Ala, and to a lesser extent of Ser and Thr, in the penultimate position of the peptide stimulates the action of the drugs. In contrast, Gly in the P or A sites of the PTC strongly counteracts the antibiotics' inhibitory effect.

Our findings reveal the most extreme manifestation of the dependency of CHL or LZD action on the nature of the PTC donor and acceptor substrates. It is very likely that the action of these antibiotics is affected by a more extended context. Thus, despite a strong preference for the presence of Ala at position (–1) in the sites of the strongest arrest, this amino acid can be found in the penultimate peptide position throughout the entire spectrum of ~60,000 codons that were analyzed in the CHL and LZD samples (Fig. S5A). Of note, the positive effect of Ala (–1) on CHL-dependent stalling at the fifth codon of the *hns* gene seems to be alleviated by the Leu5 to Ala mutation (Fig. 3D), an effect that was not observed at other examined sites. Therefore, the context in which Ala (–1) appears affects the strength of the drug-dependent translation arrest. For example, the stimulatory effect of Ala (–1) upon CHL or LZD action can be completely negated when Gly is present at the peptide's C terminus (Fig. S7A), whereas C-terminal Asp seems to additionally boost the effect of penultimate Ala, Ser, or Thr on CHL-dependent translation arrest (Fig. S7E). A more comprehensive analysis of the cumulative changes in codon-specific ribosome occupancy showed that both the stimulatory effect of Ala (–1) and the counteracting influence of Gly (0) and Gly (+1) may be modulated by the identity of the other amino acid residues in these three positions (Fig. S7A–D).

Because CHL has been traditionally viewed as a universal inhibitor of peptide bond formation, it has been used to "freeze" the ribosomes on mRNA in some profiling experiments (30, 35), an approach challenged by several studies where trends similar to the ones we presented here were noted (29, 31, 36, 37). We show that exposure of the cells to high concentrations of CHL (or LZD) does not in fact freeze the ribosomes on mRNA, but allows them to redistribute from the sites less favorable for the drug-dependent arrest to the downstream strong-arrest codons. This effect, for example, can be readily observed near the sites of translation initiation. A peak of ribosome density is prominent at the start codons of many genes in the untreated cells (30) and can be visualized by the metagene analysis (Fig. S8, gray plot). In the CHL- or LZD-treated cells, the relative overall start codon occupancy is dramatically decreased, indicative of poor ability of LZD or CHL to inhibit the first peptide bond formation and the resulting redistribution of the ribosomes from the translation initiation site to the downstream codons (Fig. S8, blue and orange plots). This observation is in line with the results of toeprinting experiments where both antibiotics failed to arrest translation at the start codon (Fig. 3 and Fig. S1). Altogether, our results strongly argue against the use of CHL in the ribosome-profiling experiments if precise position of the ribosomes on mRNA is being analyzed.

Our finding that CHL and LZD act at the defined locations within the gene extends the concept of context-specific action of ribosome-targeting inhibitors (32). Macrolides, which bind to the nascent peptide exit tunnel, arrest translation at a limited number of codons within the ORF also depending on the nature of the nascent peptide and of the incoming amino acid (27, 28, 32, 38). The context requirements for macrolides, however, are principally different from those for the PTC-targeting antibiotics. The locations where LZD or CHL would arrest translation are defined

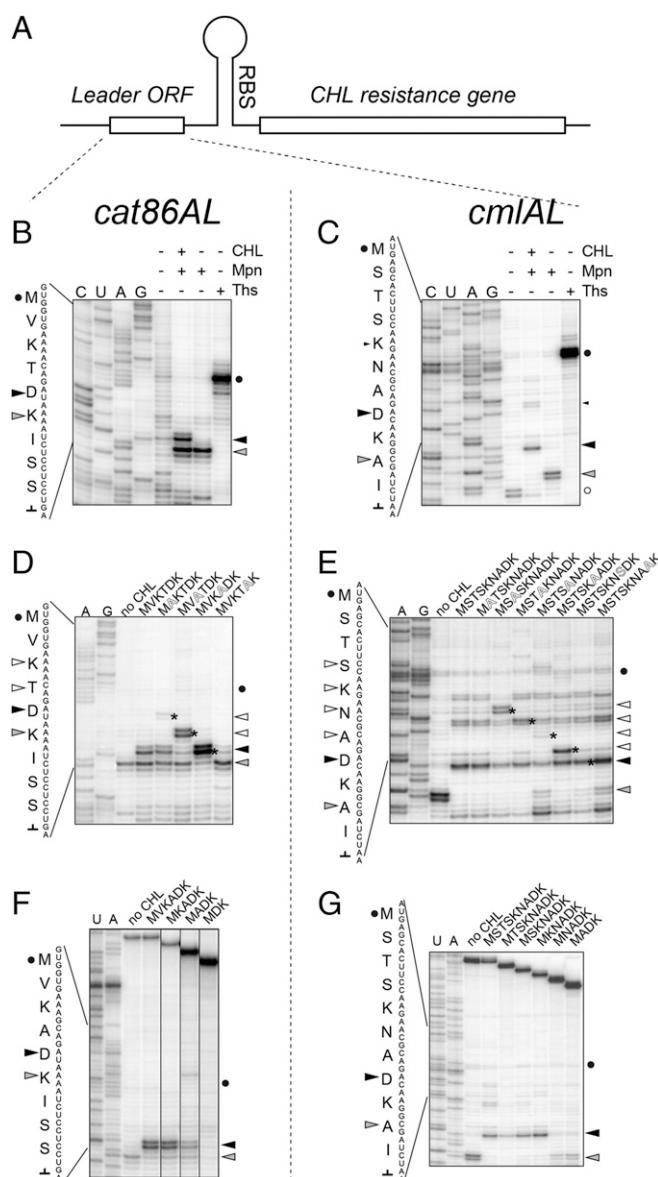


Fig. 4. CHL-induced arrest at the leader ORFs of inducible resistance genes. (A) The general organization of inducible CHL resistance genes. In the absence of antibiotic, the leader ORF is constitutively translated, but the expression of the resistance cistron is attenuated because its ribosome-binding site (RBS) is sequestered in the mRNA secondary structure. In the presence of CHL, translation of the leader ORF is arrested at a specific internal codon. Ribosome stalling mediates rearrangement of the mRNA structure resulting in activation of expression of the resistance gene. (B and C) CHL-induced translation arrest at the fifth codon of the *cat86A* leader ORF (B) or the eighth codon of the *cmlA* leader ORF (C). The control antibiotic thiostrepton (Ths) stalls translation at the start codon (black circle). A black triangle indicates the toeprint band representing CHL-induced programmed translation arrest. The gray triangle points to the band produced by ribosomes stalled before the “hungry” Ile codon [because of the presence in the reaction of mupirocin (Mpn), an Ile-tRNA synthetase inhibitor]. The band produced by ribosomes paused during termination of the *cmlAL* translation is marked with an open circle. (D and E) Alanine-scanning mutagenesis of the *cat86AL* (D) or *cmlAL* (E) alters the location of CHL-mediated translation arrest. As in B and C, the translation initiation site is marked by a circle, the site of the programmed CHL-induced arrest is shown by a black triangle, and the “Mpn band” is indicated by a gray triangle. The new sites of arrest, which appear due to the presence of new Ala residues in the mutant ORFs, are marked by the asterisks on the gel and are indicated by the open triangles. (F and G) N-terminal truncations of the *cat86AL* (F) or *cmlAL* (G) and their impact on the efficiency of CHL-induced translation arrest. The start codon, CHL band, and mupirocin band are indicated by a circle, black triangle, and gray triangle, respectively.

primarily by individual amino acids, but the sites of macrolide action are delineated by short amino acid sequences (27, 28). As a result, PTC inhibitors halt translation at multiple locations along the gene, whereas macrolides arrest ribosome only at a limited number of codons within the ORF, and synthesis of the proteins lacking the “problematic” sequences may not be inhibited at all (32, 39). For this reason, cells treated with excess of macrolide antibiotics continue to synthesize a limited subset of polypeptides (32), whereas protein translation in cells exposed to CHL or LZD is essentially abolished (Fig. S2). Nevertheless, our data suggest that CHL and LZD should be viewed as “poor” inhibitors of peptide bond formation even within the preferred context. At high drugs concentration, translation is only transiently paused at specific codons, leading to the appearance of a number of “arrest bands” in toeprinting gels, rather than at a unique stalling site (Fig. S14 and Fig. 3B).

Similar to the inducible macrolide resistance genes, induction of CHL resistance requires programmed ribosome stalling within the leader ORFs (40). Our findings revealed the principles of antibiotic-induced site-specific arrest of translation of the regulatory ORFs of two model CHL resistance genes. Translation of the *cmlAL* ORF is stalled by CHL after Ala, the amino acid most conducive to antibiotic action, appears in the penultimate position of the nascent peptide (Fig. 4C). Furthermore, synthesis of the CmlAL peptide is halted within the sequence ADK, one of the best motifs for CHL-dependent arrest (Fig. S7E). Arrest of translation of the Cat86AL leader peptide occurs within a similar context, except that Ala is replaced with Thr, another amino acid conducive to CHL action (Fig. 4C and Fig. S7E). Thus, programmed ribosome stalling responsible for induction of CHL resistance simply exploits the sequence context most favorable for the action of this antibiotic. Although the *cmlA* and *cat86* resistance genes come from evolutionarily distant bacterial species, the sites of CHL-induced ribosome stalling within the leader ORFs of these genes conform to the contexts that we identified when exploring the CHL and LZD action in *E. coli*. Therefore, we believe that the uncovered trends in the specificity of LZD and CHL action are universal, rather than strain or species specific.

We still lack the understanding of the mechanistic principles of context specificity of CHL and LZD action. Crystallographic reconstructions have uncovered how these drugs bind to the PTC of the vacant bacterial ribosome (10–12, 19, 20). However, the gained structural and functional implications are likely incomplete or possibly even misleading because our data clearly show that the key aspects of the drug binding and action must critically depend on the properties of the peptidyl nascent chain and the aminoacyl-acceptor. The effects of the PTC substrates could be direct, involving immediate interactions between the key amino acid residues and the drug, or indirect, when the properties of the drug-binding pocket are allosterically affected by the ribosomal ligands.

The context-specific action of the PTC inhibitors reflects the functional interplay between the nascent chain and the PTC, which has been demonstrated in a range of ribosomal functions: from programmed translation arrest, to termination and recoding (reviewed in ref. 41). Our previous studies (32) and the findings presented here strongly suggest that many antibiotics that directly or indirectly influence peptide bond formation, translocation of the substrates through the PTC, or egress of the nascent protein chain will be influenced by the sequence context. Improving our understanding of these principles will pave the way for the knowledge-based discovery of better protein synthesis inhibitors.

Materials and Methods

Ribosome Profiling. Ribosome profiling was carried out using the antibiotic hypersusceptible *E. coli* strain BWDK, a derivative of the strain BW25113 with inactivated *toIC* gene [F⁺, DE(araD-araB)567, lacZ4787(del)::rrnB-3, λ , rph-1, DE(rhaD-rhaB)568, hsdR514, toIC] (32). The overnight culture grown in LB medium was diluted 100-fold into three conical baffled 1-L flasks with 100 mL of LB supplemented with 0.2% glucose and grown with vigorous shaking at 37 °C. When the culture density reached OD₆₀₀ of ~0.5, CHL or LZD were added to a final concentration of 100 or 800 μ g/mL (100-fold minimum inhibitory concentration) and incubation continued for 2.5 min. The control culture did not receive a drug treatment. Cells were harvested by

rapid filtration, frozen in liquid nitrogen, and processed for ribosome profiling as described (30). The library for Illumina sequencing was prepared using RNA fragments ranging in size between 28 and 42 nt. Analysis of ribosome profiling was carried out using the GALAXY pipeline (28, 42). The first position of the P-site codon was assigned at 15 nt away from the 3'-end of the read (37). Details of analysis of the ribosome-profiling data are presented in *SI Materials and Methods*.

Generation of Templates for in Vitro Translation and Toeprinting Analysis. Preparation of DNA templates for toeprinting is described in *SI Materials and Methods*, and the primers used are listed in *Table S1*. Toeprinting reactions were carried out in 5 μ L of PURExpress transcription-translation system (New England Biolabs) as previously described (43, 44). The final concentrations of LZD, CHL, mupirocin, borrelidin, or thiostrepton in the reactions were 50 μ M, unless otherwise indicated. Gels were quantified

using ImageJ (45), and relative stalling efficiency was calculated using the following equation:

$$\text{Relative stalling efficiency} = \frac{Ab - Bd}{(Ab - Bd) + (Cc - Bd)}$$

where *Ab* is the intensity of the band representing CHL- or LZD-dependent stalling, *Cc* is the intensity of the band representing ribosome stalling at the downstream "catch" codon, and *Bd* is the background.

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