The Molecular Mechanism of Peptide-mediated Erythromycin Resistance*

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The macrolide antibiotic erythromycin binds at the entrance of the nascent peptide exit tunnel of the large ribosomal subunit and blocks synthesis of peptides longer than between six and eight amino acids. Expression of a short open reading frame in 23 S rRNA encoding five amino acids confers resistance to erythromycin by a mechanism that depends strongly on both the sequence and the length of the peptide. In this work we have used a cell-free system for protein synthesis with components of high purity to clarify the molecular basis of the mechanism. We have found that the nascent resistance peptide interacts with erythromycin and destabilizes its interaction with 23 S rRNA. It is, however, in the termination step when the pentapeptide is removed from the peptidyl-tRNA by a class 1 release factor that erythromycin is ejected from the ribosome with high probability. Synthesis of a hexa- or heptapeptide with the same five N-terminal amino acids neither leads to ejection of erythromycin nor to drug resistance. We propose a structural model for the resistance mechanism, which is supported by docking studies. The rate constants obtained from our biochemical experiments are also used to predict the degree of erythromycin resistance conferred by varying levels of resistance peptide expression in living Escherichia coli cells subjected to varying concentrations of erythromycin. These model predictions are compared with experimental observations from growing bacterial cultures, and excellent agreement is found between theoretical prediction and experimental observation.

Erythromycin is a clinically important broad-spectrum antibiotic that belongs to the macrolide class. It binds to a site in 23 S rRNA on the large ribosomal subunit (50 S) close to the peptidyl transferase center, near the entrance to the nascent peptide exit tunnel (1). Erythromycin-bound ribosomes can synthesize peptides with lengths between six and eight amino acids, but further peptide elongation is inhibited, and peptidyl-tRNA dissociates prematurely from the ribosome in the drop-off pathway (2). Different macrolides allow formation of peptides with different lengths depending on the space available between the macrolide and the peptidyl transferase center. This suggests that macrolides act by preventing the nascent peptide from entering the peptide exit tunnel in the 50 S subunit (2). Once a nascent peptide has passed the erythromycin binding site and entered the peptide exit tunnel of a drug-free ribosome, erythromycin cannot bind to the 50 S subunit, which makes the ribosome refractory to the drug until peptide elongation is terminated by a class 1 release factor (3).

The way nascent peptides interact with the exit tunnel is important both for regulation of messenger RNA (mRNA) translation and protein export (4). For example, expression of the ermC methyltransferase, which causes erythromycin resistance by methylating base A2058 (Escherichia coli numbering) at the erythromycin binding site in 23 S rRNA, is regulated by nascent peptide-erythromycin interactions in the peptide exit tunnel. That is, when there is erythromycin in a cell carrying the ermC gene, ribosomes are stalled during translation of an open reading frame present in the leader of the ermC mRNA. This causes rearrangements of the secondary structure of the leader mRNA, which makes the ribosome binding site available for initiation of translation of the ErmC encoding the open reading frame of the ermC mRNA. This regulation requires a special sequence of the leader-encoded peptide, suggesting the existence of specific interactions between the peptide, the peptide exit tunnel, and erythromycin (5).

Another example of such specific interactions is the mechanism by which expression of a small open reading frame buried in the E. coli 23 S rRNA and encoding a pentapeptide causes low level resistance to erythromycin. This pentapeptide can only work in cis, meaning that resistance is conferred only to a ribosome on which the peptide is synthesized (6). Random libraries have been used to determine a consensus sequence for peptides that cause erythromycin resistance, i.e. fMet-(bulky/hydrophobic)-(Leu/Ile)-(hydrophobic)-Val (7). The random library approach has also been used to select resistance peptides to macrolides other than erythromycin. These studies have established correlations between macrolide structures and resistance peptide sequences, suggesting a unique peptide-drug interaction in the ribosomal tunnel for each tested macrolide (8–10). It has been suggested that synthesis of the cis-acting peptide that confers resistance to erythromycin removes the drug from the ribosome in an unknown manner (8). However, there has been no direct experimental evidence to support this proposal, and the molecular mechanism by which peptide synthesis could putatively remove erythromycin from the ribosome has remained obscure.

We have used a cell-free translation system with purified components from E. coli (11) to study the mechanism of peptide-mediated erythromycin resistance. We have found that, indeed, translation of the resistance peptide mRNA ejects the peptide, and we have identified the very step where this occurs. Based on our biochemical data and with support from docking simulations, we propose a structural model for...
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Both initiation mix and recycling mix were preincubated for 10 min at 37 °C to allow for formation of ribosomal initiation complexes and ternary complexes, respectively. After mixing 10 μl of the initiation mix with 10 μl of the recycling mix, reactions were quenched at the specified time points by adding 135 μl of 20% formic acid, and peptide formation was analyzed using reverse phase HPLC as described in Tenson et al. (2).

The amount of peptide \( P \) that is produced per time unit depends on the ribosome recycling rate, \( k_1 \), and the amount of active ribosomes, \( R \),

\[
\frac{dP}{dt} = k_1 R
\]

(Eq. 1)

Without josamycin, which inhibits formation of these short peptides, the amount of active ribosomes is constant, \( R = R_{tot} \), and the recycling rate can be determined from the slope of a curve where the amount of peptides is plotted versus time, \( P = R_{tot} k_1 t \).

However, if erythromycin is chased with a large excess of josamycin when an erythromycin molecule dissociates from the ribosome, it is immediately replaced by a josamycin molecule, which shuts down protein synthesis. Accordingly, the amount of active ribosomes becomes

\[
R = R_{tot} e^{-k_2 t}
\]

(Eq. 2)

where \( k_2 \) is the rate constant for erythromycin dissociation. Insertion of Equation 1 in Equation 2 leads to the differential equation

\[
\frac{dP}{dt} = k_1 R_{tot} e^{-k_2 t}
\]

(Eq. 3)

for peptide synthesis, which has the solution

\[
P = R_{tot} \frac{k_1}{k_2} (1 - e^{-k_2 t})
\]

(Eq. 4)

Using the \( k_1 \) value estimated from the experiment performed in the absence of josamycin, the parameter \( k_2 \) was varied to fit the Equation 4 model to experimental data with the help of the Marquardt algorithm (16) implemented in Origin 7 (OriginLab Corp.).

Erythromycin and Peptide Dissociation Rates

The mixture for initiation of protein synthesis contained ribosomes (1.4 μM, ~70% active), \([^{1}H]fMet-tRNAfMet (1 μM), mRNA (2.5 μM), IF2 (0.5 μM), IF1 (1 μM), IF3 (1 μM), and, when relevant \([^{14}C]\)erythromycin (2 μM). The protein elongation mixture contained EF-G (1.6 μM), EF-Tu (40 μM), EF-Ts (1 μM), and tRNA bulk (~0.18 μM), inorganic pyrophosphatase (5 μg/ml), myokinase (3 μg/ml), pyruvate kinase (50 μg/ml). Elongation was halted at the desired peptide lengths by exclusion of the amino acid and the aminoacyl-tRNA synthetase necessary to form the ternary complex that was reading next downstream codon. The concentrations of the added aminoacyl-tRNA synthetase were (0.15 units/μl) (defined in Ehrenberg et al. (15)), and the added amino acids were (alanine 1.5 mM, leucine 300 μM, and 100 μM concentrations of each of the others). Josamycin (165 μM) was also added to the recycling mix when relevant.

Recycling Experiments

The initiation mixture contained ribosomes (0.24 μM, ~70% active), \([^{1}H]fMet-tRNAfMet (5 μM), mRNA (0.5 μM), IF2 (0.5 μM), IF1 (1 μM), IF3 (1 μM), and erythromycin (0.6 μM) in the chase and 6 μM in the experiments with only erythromycin). The recycling mixture contained EF-G (2 μM), EF-Tu (40 μM), EF-Ts (1 μM), RF2 (2 μM), RF3 (2 μM), RF7 (2 μM), tRNA bulk (~0.18 μM), inorganic pyrophosphatase (5 μg/ml), myokinase (3 μg/ml), pyruvate kinase (50 μg/ml), the relevant aminoacyl-tRNA synthetases (0.15 units/μl) (defined in Ehrenberg et al. 15), and amino acids (alanine 1.5 mM, leucine 300 μM, and 100 μM concentrations of each of the others). Josamycin (165 μM) was also added to the recycling mix when relevant.
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mix with 20 μl of the elongation mix, reactions were quenched by the addition of 1 ml of ice-cold polyvinyl and applied to nitrocellulose filters. The filters were washed twice with 1 ml of polyvinyl, and both the 3H and 14C activity were counted for both the flow-through and the filters.

**Formic Acid Precipitation Assay**—After mixing 20 μl of the initiation mix with 20 μl of the elongation mix, reactions were quenched by the addition of 150 μl of 20% formic acid, and the precipitates were pelleted by centrifugation. The 3H activities in the supernatants, containing released peptides were counted directly, whereas 160 μl of 0.5 mM KOH was added to the pellets. After 10 min of incubation at room temperature, 10 μl of 100% formic acid was added, and the precipitates were pelleted again. The 3H activities in the supernatants at this second step correspond to the peptides that were still bound to tRNA at the time point when the reaction was quenched.

**Docking of the Peptides to the Ribosome**

Computational modeling was done to investigate the possible modes of interaction between peptides and erythromycin in different stages of peptide elongation. We used docking of the resistance peptide, fMet-Arg-Leu-Phe-Val-Stop (fMLRFV), to find a specific pattern of interaction with erythromycin. Furthermore, we used the peptide fMet-Asn-Ala-Ile-Lys-Stop (fMNAIK) as the negative control, in line with the experimental work (9). For this purpose we used GOLD 3.0 (CCDC, Cambridge, UK) in combination with the crystal structure 1QVG (17). The docking study was carried out using 2,000,000 operations per docking. Atom c21 in erythromycin was defined as the active site, and a radius of 10 Å was used in the floodfill. Thus an “active site” was defined around the erythromycin facing the A and P sites. The two peptides were docked as tri-, tetra-, pentapeptide (extended with Ala). Each peptide was docked 20 times, and the 15 best solutions were saved. We used the Chemscore algorithm (16) implemented in Origin 7 (OriginLab Corp.).

**Results**

**Simulations of Peptide-mediated Resistance in the Living Cell**

Based on our model for erythromycin ejection from the ribosome and biochemical data, we set up a system of differential equations of ribosome systems. Real-time PCR was run and monitored in Rotor-Gene 5.0.47. The transcription real-time PCR program was as follows; 1) annealing of the forward primer to mRNA (75 °C for 2 min, 65 °C for 5 min, and 53 °C for 5 min); 2) reverse transcription reaction, started by adding TaqMan reverse transcription buffer, dNTPs, RNAse inhibitor, and reverse transcriptase followed by incubation at 45 °C for 10 min, 48 °C for 30 min, and 95 °C for 30 min; 3) real-time PCR, started by the addition of PCR buffer, dNTP, AmpliTaq Gold DNA polymerase, and the respective reverse primers and Taqman probes followed by PCR steps (prePCR (50 °C for 2 min and 95 °C for 10 min) and 40 PCR cycles (95 °C for 15 s, 50 °C for 30 s, and 60 °C for 15 s). The final reaction volume was 25 μl. The concentrations of forward and reverse primers were 900 nM each, and the probe concentration was 100 nM. Annealing and reverse transcription steps were done in GeneAmp PCR System 2700 (Applied Biosystems). The real-time PCR was run and monitored in Rotor-Gene 5.0.47.

**Primers**—Specific primers for resistance peptide encoding mRNA were: forward, d(AAAAGGCCGCTTATTAGG), reverse, d(TGTGACTCTT- TAAGGAGTTTACAT), and Taqman probe, d(CATAGAGATTTCA- GCTAGTTAAAACAAACAAAAACCA). Specific primers for EF-Tu mRNA were: forward, d(GAGATGGAGAATACGTCTTG), reverse, d(AC- CAGGCGTGCATTG), and Taqman probe, d(CGCGCCGACAG- GACGGCCTT). Taqman probes had the 5’ end modified with a FAM fluorophore and the 3’ end modified with a TAMRA fluorophore.

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Stoichiometric Removal of Erythromycin by Resistance Peptide Synthesis—To study the effects of resistance peptide synthesis on the rate of dissociation of erythromycin from the 50 S subunit, we took advantage of a cell-free translation system with purified components from E. coli (11). The resistance pentapeptide fMLRFV and a control pentapeptide, fMNAIK, were synthesized (9) on ribosomes in recycling mode (19). Erythromycin insignificantly affected the rate of synthesis of resistance and control peptide, whereas their synthesis was shut down by the presence of josamycin (Fig. 1). We took advantage of this by chasing the erythromycin, originally on the recycling ribosomes, with josamycin (Fig. 1). Because the two drugs have overlapping ribosomal binding sites (1, 20, 21), josamycin cannot bind and shut down peptide synthesis until after dissociation of erythromycin from the 50 S subunit. The josamycin concentration used in the chase (83 μM) leads to an association rate of 2.7 s⁻¹ (21). Thus, the rate-limiting step in the josa-
Erythromycin-induced inhibition of peptide formation is the erythromycin dissociation. The value of the rate constant for dissociation of erythromycin from ribosomes synthesizing the control peptide was estimated as 0.01 s\(^{-1}\) (Fig. 1B), which corresponds to the rate constant for spontaneous dissociation of erythromycin from empty ribosomes (21). In contrast, the value of the rate constant for dissociation of erythromycin from ribosomes synthesizing the resistance peptide was estimated as 0.03 s\(^{-1}\), a value coinciding with the rate (s\(^{-1}\)) of pentapeptide synthesis per ribosome in the absence of josamycin (Fig. 1A). From these results follows that erythromycin was removed with high probability from the ribosome during each cycle of resistance, but not control peptide synthesis. Identification of the step at which drug dissociation was induced by the cis-acting peptide required further experiments, to be described in the next paragraph.

**Dissociation of Erythromycin during Different Stages of Resistance Peptide Synthesis**—To estimate the rate constants for dissociation of erythromycin at different stages of resistance peptide synthesis, we used nitrocellulose filtration techniques.

Ribosomes were initiated for synthesis of resistance (MRLFV) or control (MNAIK) peptides. By selective exclusion of amino acids and aminoacyl-tRNA synthetases in the peptide elongation assays, ribosomes carrying fMR, fMRL, fMRLF, or fMRLF as well as fMN, fMNA, fMNAIK, or fMNAIK peptides ester-bonded to the P-site tRNA were produced. Subsequently, \(^{14}C\)erythromycin was chased from each of these ribosome complexes by the addition of unlabeled erythromycin in excess, and the fraction of \(^{14}C\)erythromycin-containing ribosomes was monitored by nitrocellulose filtration at different incubation times. From these data, rate constants for the dissociation of erythromycin were estimated, and the results are summarized in Table 1. The rate constant for dissociation of erythromycin increased from its smallest value (0.004 s\(^{-1}\)) in the initiation complex with every amino acid that was added, in accordance with the resistance peptide sequence to its largest value of 0.068 s\(^{-1}\) when the pentapeptide was completed (Fig. 2A and Table 1). There was at the same time little effect on the rate of erythromycin dissociation by amino acid addition, in accordance with the control peptide sequence (Fig. 2B and Table 1).

It has been shown that active resistance peptides must have lengths between four and six amino acids (7). To clarify why this is so, we prepared mRNAs encoding the hexapeptide fMet-Arg-Leu-Phe-Val-Ala-Stop, which is the resistance peptide with a C-terminal addition of Ala, and the heptapeptide fMet-Arg-Leu-Phe-Val-Ala-Asn-Stop, which is the resistance peptide with a C-terminal addition of Ala-Asn. Both these C-terminal additions reduced the rate constant for erythromycin dissociation from 0.068 s\(^{-1}\) (in the presence of the authentic resistance peptide) to 0.014 s\(^{-1}\) (in the presence of the C-terminal extension) (Table 1).

These results show that when the resistance peptide grew from two to five amino acids, this led to successively faster dissociation of erythromycin. Because, however, synthesis of the resistance peptide was considerably faster than the largest rate of erythromycin dissociation, these data cannot explain why every round of resistance peptide synthesis resulted in near-stoichiometric removal of the 50 S-bound erythromycin (Fig. 1). This pointed at class 1 release factor-induced peptide release in the case of the control peptide (Fig. 2). The pentapeptide synthesis rate per ribosome in the absence of josamycin (Fig. 1B) is 0.03 s\(^{-1}\) (3 pmol min\(^{-1}\)/1.7 pmol ribosomes = 1.8 min\(^{-1}\) × 0.03 s\(^{-1}\)).

**TABLE 1**

| Translated peptide | Erythromycin dissociation rate constant (s\(^{-1}\)) |
|--------------------|-----------------------------------------------|
| Initial complex    | 0.011 ± 0.001                                 |
| MR                 | 0.017 ± 0.005                                 |
| MRL                | 0.025 ± 0.004                                 |
| MRLF               | 0.051 ± 0.004                                 |
| MRLFV              | 0.068 ± 0.006                                 |
| MRLFVA             | 0.014 ± 0.001                                 |
| MRLFVAN            | 0.014 ± 0.001                                 |
| MN                 | 0.011 ± 0.001                                 |
| MNA                | 0.015 ± 0.001                                 |
| MNAI               | 0.016 ± 0.001                                 |
| MNAIK              | 0.017 ± 0.001                                 |

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The largest rate constant for erythromycin dissociation (0.14 s\(^{-1}\)) was obtained when either one of the class 1 release factors was also present to terminate the synthesis of the resistance peptide at the UAA codon of its mRNA (Fig. 2C, Table 2). At the same time, there was no effect on the rate of erythromycin dissociation by release factor addition in the case of the control peptide (Fig. 2C, Table 2). To further investigate class 1 release factor action, we used nitrocellulose binding to monitor the release of different peptides from the ribosome and formic acid precipitation followed by peptide identification by HPLC to directly monitor hydrolysis of the ester bond connecting peptide and P-site tRNA. The rate of dissociation from the ribosome and the rate of ester bond hydrolysis were similar in the cases described below in this section of text, suggesting fast dissociation of peptides from the ribosome after the rate-limiting ester bond hydrolysis.

The rate of resistance peptide release from the ribosome, as induced by either one of the class 1 release factors (0.073 s\(^{-1}\)) as monitored by the ribosome-bound \(^3\)H-labeled fMet, was significantly smaller than the rate constant for dissociation of erythromycin (0.14 s\(^{-1}\)). At the same time, the rate of control peptide release as induced by RF2 (0.22 s\(^{-1}\)) was almost 30 times larger than the rate constant for erythromycin dissociation (Fig. 2E). These results in conjunction with the observation (Fig. 1) that every cycle of resistance peptide synthesis removed the ribosome-bound erythromycin with high probability suggest, first, that binding of a class 1 release factor to an erythromycin-containing ribosome carrying a resistance pentapeptide further destabilized the binding of the...
drug to the ribosome and, second, that termination was slow enough to allow dissociation of erythromycin from the ribosome with a probability close to one, in accordance with the results in Fig. 1. It cannot be excluded that dissociation of erythromycin was strictly required for termination to occur, in which case the probability for drug rejection would be exactly 100%. The reason for the ambiguity relates to the experimental design in which $[14C]$erythromycin was chased with unlabeled erythromycin at a high concentration (75 μM), which could allow for rapid rebinding of an unlabeled erythromycin after dissociation of the labeled one (21), before significant termination could occur. In this latter scenario, which leads to the simplest interpretation of the peptide release data, termination in our in vitro experiments occurred in the presence of erythromycin.

The addition of RF2 to ribosomes carrying the resistance peptide with a C-terminal addition of one amino acid (the hexapeptide) led to peptide release with a rate constant of 0.015 s$^{-1}$, virtually identical with the rate constant of 0.014 s$^{-1}$ for dissociation of erythromycin (Figs. 2, D and E, and Table 2). The addition of RF2 to the resistance peptide with a C-terminal addition of two amino acids (the heptapeptide) led to peptide release with a considerably smaller rate constant of 0.006 s$^{-1}$ but to a similar rate constant of 0.014 s$^{-1}$ for dissociation of erythromycin (Figs. 2, D and E, and Table 2). This rate constant for dissociation of erythro-
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Erythromycin is similar to the corresponding rate constant for the peptide-lacking initiation complex. These experiments show that release of the extended peptides did not accelerate dissociation of erythromycin, in line with the previous observation of a strong sequence length dependence of resistance activity (7).

Treatment of ribosomes carrying full-length resistance peptides with puromycin, an antibiotic mimicking the aminoacylated 3′-adenosine of an aminoacylated tRNA (17), did not alter the rate of dissociation of erythromycin (Fig. 2D and Table 2). It was not possible to monitor release of the resistance peptide-puromycin complex from the ribosome, since it remained filter-bound in free as well as ribosome-bound configuration. We could, however, monitor peptidyl transfer to puromycin using HPLC after formic acid precipitation. We found that the rate constant for transfer of the resistance peptide to puromycin (0.23 s\(^{-1}\)) was much larger than the rate constant for dissociation of erythromycin (0.067 s\(^{-1}\)) (Figs. 2, D and F). This means that transfer of the resistance peptide to puromycin was unhindered by the presence of erythromycin, in contrast to the hydrolytic reaction induced by a class 1 release factor (Fig. 2C and Table 2). It is normally assumed that when a small peptide is transferred to puromycin, it rapidly leaves the ribosome. However, if this were the case, one would expect that the rate constant for erythromycin release would be reduced from its value of 0.068 s\(^{-1}\) in the absence of puromycin to its value of 0.011 s\(^{-1}\) in the absence of peptide. The experiments show, in contrast, that in response to puromycin treatment dissociation of erythromycin remained unaltered at 0.067 s\(^{-1}\). This suggests that the resistance peptide-puromycin complex remained ribosome-bound long enough to allow for dissociation of the radio-labeled erythromycin.

Docking of the Resistance Peptide to the Ribosome—Previous genetic studies (7) and the biochemical data in this work suggest the existence of specific interactions between the resistance peptide and ribosome-bound erythromycin. To test this, we performed docking simulations with a resistance or a control peptide anchored to an A-site- or a P-site-bound tRNA of a ribosome in complex with erythromycin. In 8 of the top 15 simulations for the resistance pentapeptide anchored to the P-site tRNA, the leucine in fMRLFV was bound to a small hydrophobic cavity on the surface of erythromycin, between the cladinosine and desosamine residues (see Fig. 4, E and F), and a similar result was obtained for the tetrapeptide fMRLE. Similar, but less pronounced leucine binding patterns were observed also for fMRLFV and fMRLE anchored to the A-site tRNA. At the same time, no distinct binding patterns were observed for amino acids other than leucine in the resistance peptide or for any of the amino acids in the control peptide fMNAIK. In the case of the resistance tripeptide, the leucine did not reach into the erythromycin cavity, and in the case of the resistance hexapeptide, the leucine binding pattern was gone, possibly due to steric hindrance.

Validation of the Model for Resistance Peptide Action by Cell Population Experiments—From the biochemical experiments described above, kinetic constants for resistance peptide action were obtained (Table 2). We constructed a model for erythromycin resistance in bacterial populations (Fig. 3A) based on these and other parameters (listed in the supplemental material) for protein synthesis obtained from our cell-free mRNA translation system (21). The model (detailed description in the supplemental material) contains seven different states of the large ribosomal subunit (50 S) (Fig. 3A); it accounts for dilution of all compounds due to cell volume growth and for a finite rate of diffusion across the cell membrane, which reduces the intracellular concentration in relation to the outer concentration of erythromycin. Furthermore, the model takes into account the efflux pumps used by E. coli to actively transport erythromycin and other antibiotic drugs from the membrane and cytoplasm to the growth medium (22).

To validate the model, we varied the expression of rpmRNA under tac promoter control from a multicopy plasmid by varying the concentration of IPTG in an erythromycin-containing growth medium (6, 9). Cell growth at different IPTG and erythromycin concentrations was monitored along with reverse transcription real-time PCR analysis of the intracellular concentration of rpmRNA relative to the concentration of EF-Tu mRNA (Fig. 3C, inset). The tac promoter was leaky, and the response in rpmRNA synthesis to the external IPTG level was linear. At the highest IPTG concentration (600 µM) in the medium, the mRNA level was 3-fold higher than in the absence of IPTG (Fig. 3C, inset). Increasing IPTG concentrations led to increasing erythromycin resistance until a plateau, specific for each concentration of erythromycin, was reached (Fig. 3C). The increase in bacterial mass (optical density) during 8 h of growth at varying concentrations of erythromycin and IPTG in the medium were monitored, and there was excellent agreement between the experimentally observed and model-simulated growth behavior in which the measured, relative rpmRNA levels had been taken into account (Fig. 3B).

DISCUSSION

Erythromycin binds in the nascent peptide exit tunnel close to the peptidyl transferase center (1, 23) (Fig. 4A) and prevents synthesis of peptides longer than eight amino acids (2). Expression of a mini-gene buried in the 23 S rRNA causes low level resistance to erythromycin (6), and it has been suggested that synthesis of this resistance peptide on an erythromycin-containing ribosome can clean it from the drug, thereby making an erythromycin-free 50 S subunit available for a new round of initiation of protein synthesis with another mRNA (8). When the nascent peptide is longer than six to eight amino acids, it covers the erythromycin binding site, which makes the ribosome refractory to further inhibition by erythromycin, allowing for synthesis of full-length proteins (3, 21).

The present experiments directly demonstrate that synthesis of a resistance peptide can, indeed, remove erythromycin from the 50 S subunit. During every cycle of resistance peptide synthesis erythromycin dissociates with close to 100% probability, whereas the synthesis of a control peptide does not induce dissociation of the drug (Fig. 1). As the resistance peptide grows by successive amino acid additions, the rate constant for dissociation of erythromycin increases in a stepwise manner (Table 1). It is, however, not until class 1 release factor induced termination of the full-length resistance pentapeptide, that erythromycin is removed from the ribosome with high probability. Termination is, in other words, the crucial kinetic step for erythromycin dissociation and, therefore, the point at which resistance is conferred.

To validate the mechanism for resistance peptide action, we modeled it in the context of the cytoplasm of a living cell (Fig. 3A and supplemental material) using kinetic data from the present (Table 2) and earlier (21) work. We describe in particular the degree to which inhibition of the growth rate of a bacterial population due to the presence of varying concentrations of erythromycin in the cytoplasm is expected to be relieved by the expression of the resistance peptide at varying levels (Fig. 3B).

These simulations were compared with experimental observations from a bacterial population containing the resistance peptide gene under tac promoter control on a multicopy plasmid. The cells were grown in media containing varying concentrations of erythromycin as well as IPTG to control the level of resistance peptide expression. The increase in bacterial mass during 8 h of growth was monitored by optical
density (Fig. 3C) along with the level of resistance peptide mRNA normalized to the level of EF-Tu mRNA (Fig. 3C, inset), as measured by reverse transcription real-time PCR. The simulated growth rates in Fig. 3B, where the experimentally measured resistance peptide mRNAs are taken into account, are in excellent agreement with the measured growth rates in Fig. 3C. This shows that the mechanism we propose for peptide-mediated low level resistance against erythromycin (Fig. 3A) and the rate constants obtained from our cell-free in vitro translation system (Table 2 and Ref. 21) are sufficient to fully account for the in vivo induced resistance in a large interval of erythromycin concentrations and peptide expression levels (Figs. 3, B and C (and inset)).

The structural basis of resistance peptide action is of considerable interest, not the least because it is one special case of the general and poorly understood phenomenon of peptide-specific interactions with the ribosomal peptide exit tunnel (4, 24). Our data show that when the control peptide grows from a di- to a pentapeptide, there is little change in the rate constant for erythromycin dissociation. When, furthermore, RF2 is added to terminate peptide synthesis, hydrolysis of the ester bond in the peptidyl-tRNA proceeds with the same rate as in the absence of erythromycin (Tables 1 and 2). For the resistance peptide, in contrast, our data show that the rate constant for erythromycin dissociation increases gradually by a factor of six as the peptide grows from just the fMet to di- and then to pentapeptide. In addition, when RF2 is added, the rate constant for erythromycin release is further enhanced by a factor of two, and the rate of ester bond hydrolysis is much smaller than in the absence of erythromycin (Tables 1 and 2). We know from data obtained from open reading frame libraries that the consensus sequence for peptide-mediated erythromycin resistance has two outstanding features; there is a leucine or isoleucine in the third position and a valine in the fifth, C-terminal position (7, 9). Resistance peptides for different
types of macrolides have different consensus sequences, suggesting specific and perhaps direct interactions between the conserved residues and each type of ribosome bound macrolide (9, 10). From the present kinetic data (Tables 1 and 2) and docking simulations (Fig. 4) along with previous open reading frame library data (7), we propose a structural model for peptide-mediated erythromycin resistance (Figs. 4, A–D).

Our docking studies based on the crystal structure of a Haloarcula marismortui 50 S subunit in complex with erythromycin (1) suggest that the side chain of leucine in the resistance peptide binds to the hydrophobic cleft between the two sugar moieties of erythromycin (Figs. 4, E and F). To date, there is no crystal structure of an E. coli 50 S subunit in complex with erythromycin, but the similarity of the 50 S subunits from the two organisms near the erythromycin binding site (1, 25) suggests that our docking data are relevant also for the erythromycin-bound E. coli ribosome. Leucine binding to erythromycin is observed both for resistance tetra- and pentapeptides, and the binding pattern is more distinct for resistance peptides anchored to the P-site than to the A-site tRNA. By hypothesis, the observed interaction between the resistance tetrapeptide and the drug weakens the affinity of erythromycin for the ribosome, which accounts for the fact that a leucine (or an isoleucine) is critical for resistance peptide action. Completion of the resistance pentapeptide by the addition of valine further increases the erythromycin dissociation rate constant, probably because the force by which the resistance peptide pushes erythromycin out from its binding site increases (Fig. 4B). When a class 1 release factor binds to the pre-termination ribosome containing a resistance pentapeptide ester-bonded to the P-site tRNA, the rate constant for erythromycin dissociation increases by another factor of two. At the same time, the rate constant for ester bond hydrolysis of the peptidyl-tRNA decreases very significantly (Table 2), which partially accounts for the fact that every cycle of peptide synthesis led to erythromycin dissociation with near 100% probability (Fig. 1). It is, furthermore, possible that the resistance peptide forms a specific hydrophobic structure that prevents it from leaving the ribosome through the peptidyl transferase center after its release from the P-site tRNA. The peptide is then forced to leave the ribosome through the peptide exit tunnel, where its hydrophobic C terminus could interfere with the hydrophobic interactions between erythromycin and the exit tunnel wall and chase the drug out through the L4/L22 constriction in the tunnel (Fig. 4C). This would lead to 100% probability of drug ejection per cycle of resistance peptide synthesis. When instead of termination, an additional amino acid is added to the resistance pentapeptide, our simulations suggest that the leucine interaction with erythromycin becomes lost and that, accordingly, the hexa-peptide is expected to behave like any other peptide. It will fill up the space available between drug and peptidyl transfer center until further protein synthesis is inhibited by crowding (Fig. 4D).
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