Resistance to ketolide antibiotics by coordinated expression of rRNA methyltransferases in a bacterial producer of natural ketolides

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Edited by Chaitan Khosla, Stanford University, Stanford, CA, and accepted by the Editorial Board September 4, 2015 (received for review June 22, 2015)

Ketolides are promising new antimicrobials effective against a broad range of Gram-positive pathogens, in part because of the low propensity of these drugs to trigger the expression of resistance genes. A natural ketolide pikromycin and a related compound methymycin are produced by *Streptomyces venezuelae* strain ATCC 15439. The producer avoids the inhibitory effects of its own antibiotics by expressing two paralogous rRNA methylase genes *pikR1* and *pikR2* with seemingly redundant functions. We show here that the PikR1 and PikR2 enzymes mono- and dimethylate, respectively, the N6 amino group in 23S rRNA nucleotide A2058. PikR1 monomethylase is constitutively expressed; it confers low resistance at low fitness cost and is required for ketolide-induced activation of *pikR2* to attain high-level resistance. The regulatory mechanism controlling *pikR2* expression has been evolutionary optimized for preferential activation by ketolide antibiotics. The resistance genes and the induction mechanism remain fully functional when transferred to heterologous bacterial hosts. The anticipated wide use of ketolide antibiotics could promote horizontal transfer of these highly efficient resistance genes to pathogens. Taken together, these findings emphasized the need for surveillance of *pikR1/pikR2*-based bacterial resistance and the preemptive development of drugs that can remain effective against the ketolide-specific resistance mechanism.

ribosome | antibiotics | ketolides | resistance | macrolides

The prototypes of most of the clinically useful antibiotics, including the diverse group of protein synthesis inhibitors, have been discovered among the secondary metabolites of bacterial species. Antibiotic-producing bacteria have developed an array of resistance genes to avoid committing suicide (1). The wide medical use of antibiotics has created a strong selective pressure for such resistance genes to transfer and integrate into the genomes of bacterial pathogens, curbing the beneficial effects of the drugs and shortening their clinical lifespan. Consequently, antibiotic producers are not only our allies in providing useful drugs but also, play an adversary role by facilitating the spread of resistance.

Macrolides are among the most medically successful antibiotics originating from the secondary metabolites of actinomycetes. They inhibit translation by binding in the nascent peptide exit tunnel (NPET) close to the peptidyl transferase center (PTC) of the large ribosomal subunit (2–4). The most common mechanism of macrolide resistance involves dimethylation of an rRNA nucleotide in the drug binding site (A2058 in the *Escherichia coli* 23S rRNA) by Erm methyltransferases (5). In the absence of antibiotic, A2058 dimethylation is deleterious for the cell, because modification of a residue in a functional site of the NPET distorts production of a subset of proteins (6). Therefore, to reduce the fitness cost associated with resistance, expression of the *erm* genes is often inducible and activated only when antibiotic is present. Such induction operates through antibiotic-controlled ribosome stalling at an upstream leader ORF (5, 7).

Macrolide antibiotics are built on a 14- to 16-atom macrolactone ring decorated with various side chains. The prototype 14-atom ring macrolide erythromycin (ERY) and its second generation derivatives carry cladinose at the C3-hydroxyl position of the ring and a desosamine sugar linked to the C5-hydroxyl group (Fig. 1*B*). In the newest generation of drugs, the ketolides, the C3-cladinose is replaced with a keto function. Ketolides are viewed as one of the most promising classes of antibiotics presently under development and offer broad medical application (8). The first medically useful ketolide telithromycin (TEL) and newer ketolides currently in clinical trials show dramatically improved antibacterial activity compared with earlier generations of macrolides, and to a large extent, because of their reduced propensity to activate inducible resistance genes (9, 10).

All of the clinically relevant ketolides are synthetic or semisynthetic derivatives of natural macrolides. The only naturally occurring 14-atom macrolactone ring ketolide that is presently known is pikromycin (PKM) (11) produced by *Streptomyces venezuelae* (strain ATCC 15439) (Fig. 1). The biosynthesis pathway of PKM is unique because of a modular polyketide synthesis skipping mechanism that can divert the pathway toward production of a second macrolide molecule, methymycin (MTM), which has a smaller macrolactone ring of 12 atoms (Fig. 1*B*) (12, 13). Although both PKM and MTM possess antibacterial activity, their modes of binding to the ribosome and their mechanisms of action remain unclear.

The modular polyketide synthesis genes *pikAI-pikAV* in the biosynthetic cluster of *S. venezuelae* ATCC 15439 are preceded by

Significance

Studies of antibiotic resistance are usually initiated in earnest only after resistance has become established in clinical pathogens. Here, we forewarn of a resistance mechanism to the novel antibiotics ketolides, which are only coming into broad medical practice. We show that the balanced activities and coordinated expression of two genes, *pikR1* and *pikR2*, provide efficient protection to *Streptomyces venezuelae*, a bacterial producer of natural ketolides. Expression of the more potent gene, *pikR2*, is supported by *pikR1* and specifically induced by ketolides. The resistance mechanism remains fully functional when *pikR1* and *pikR2* are transferred to other bacterial species and affords protection against clinical ketolides. These findings emphasize the need for the preemptive development of antibiotics that can overcome this resistance mechanism.

Author contributions: A.S.M. designed research; M.M.A. and S.R. performed research; S.R.P. and D.A.H. contributed new reagents/analytic tools; M.M.A., N.V.-L., S.D., D.H.S., and A.S.M. analyzed data; and M.M.A., S.D., D.H.S., and A.S.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. C.K. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1512090112/-/DCSupplemental.

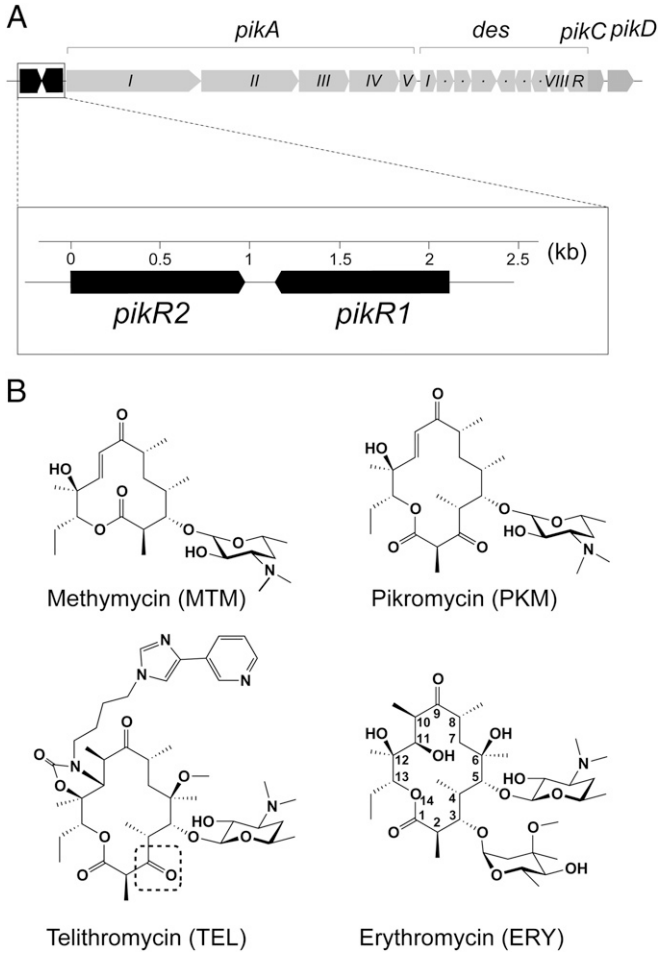

Fig. 1. *S. venezuelae pikR* resistance genes and ketolide antibiotics. (*A*) The structure of the MTM/PKM biosynthetic gene cluster in *S. venezuelae* ATCC 15439. The polyketide synthase (*pikA*) and desosamine biosynthesis (*des*) gene operons along with the *pikC* and *pikD* genes are required for production of the active MTM and PKM antibiotics. The putative resistance genes *pikR1* and *pikR2* precede the MTM/PKM biosynthesis operon. (*B*) Structures of the natural antibiotics MTM and PKM produced by *S. venezuelae* ATCC 15439, the semisynthetic clinical ketolide TEL, and the C3-cladinose containing macrolide ERY. The dotted box in the TEL structure highlights the keto group, which replaces the cladinose sugar.

two putative resistance genes, *pikR1* and *pikR2*, arranged tail to tail (Fig. 1*A*). The protein products PikR1 and PikR2 are 42% identical (Fig. S1) and show similarity to Erm-type rRNA methyltransferases (e.g., 30% and 28% identities with ErmE for PikR1 and PikR2, respectively) (14, 15). Given the significant fitness cost associated with *erm*-based resistance (6), the duplication of PikR enzymatic function in *S. venezuelae* is puzzling. Other than being of general biological interest, the mechanism by which a natural producer of ketolides attains resistance is of significant medical importance. Not in the least, it can be envisioned that selective pressure imposed by broad medical use of antibiotics could promote the transfer of natural resistance genes to pathogens. Understanding the function and regulation of *pikR1*/*pikR2* and similar genes would facilitate their surveillance and possibly, help curb their dissemination. Study of *pikR1*/*pikR2*-like genes might, in turn, stimulate preemptive development of drugs that retain antimicrobial activity against strains harboring these resistance mechanisms.

In this paper, we show that the *pikR1* and *pikR2* genes render the ketolide-producing *S. venezuelae* cells resistant to PKM and MTM as well as clinical ketolide antibiotics. Having determined the target, the mode of action, and the regulation of these genes, we reveal how *S. venezuelae* achieves ketolide resistance at a low fitness cost by balancing the activities of PikR1 and PikR2 and timing their expression. We present evidence that the expression of *pikR2* gene has been optimized through evolution to respond to ketolide antibiotics. Finally, we show that transfer of *pikR2* to other bacteria renders them resistant to ketolides, and this effect is accentuated in combination with *pikR1*. These findings illuminate resistance mechanisms that could potentially be acquired by clinical pathogens on launching new ketolide antibiotics.

Results

***pikR1* and *pikR2* Confer Resistance to MTM and PKM.** We first assessed whether *pikR1* and *pikR2*, which show similarity to macrolide resistance *erm* genes (16), confer resistance to the natural ketolides MTM and PKM produced by *S. venezuelae*. The *pikR* ORFs were individually expressed from plasmids pPikR1 and pPikR2 (Table S1) in an *E. coli* strain hypersusceptible to macrolides. Although cells lacking these genes were completely inhibited by 4 μg/mL MTM or 8 μg/mL PKM, expression of *pikR1* or *pikR2* rendered *E. coli* resistant to >512 μg/mL either of the compounds. These data indicate that pikR1 and *pikR2* confer resistance to natural ketolides and thus, have evolved or were acquired to protect *S. venezuelae* from its endogenous antibiotics.

PikR1 and PikR2 Modify the Same 23S rRNA Nucleotide. The presence of two resistance genes of potentially similar function may suggest that one of them is redundant, prompting us to dissect their individual functions. The binding sites of MTM and PKM in the bacterial ribosome have not been biochemically defined previously. However, structural evidence from the *Deinococcus radiodurans* large ribosomal subunit suggests that MTM binds in the PTC (17) rather than in the NPET, where all conventional macrolides and ketolides bind (2–4). Therefore, the site of action and the nature of the modifications introduced by PikR1 and PikR2 could not be predicted with any certainty. The majority of the investigated Erm-type enzymes confer resistance to macrolides by dimethylating the exocyclic amine of A2058 in the 23S rRNA (*E. coli* numbering throughout) (5). A2058 dimethylation (but not monomethylation) stalls the progress of reverse transcriptase (RT) on the RNA template and is readily detected by primer extension. A strong band corresponding to RT pausing at A2058 on rRNA extracted from *E. coli* cells expressing PikR2 indicated that it probably dimethylates this nucleotide (Fig. 2*A* and Fig. S2*A*). In contrast, under the standard primer extension conditions, no RT stop was detected at either A2058 or any other site within the PTC and the NPET on rRNA from cells with pPikR1 (Fig. 2*A*). However, when we optimized primer extension conditions for detecting N6 monomethylation of adenosine (*Materials and Methods* and Fig. 2*B*), RT pausing at A2058 was observed on rRNA from cells expressing PikR1 (Fig. 2*B*, lane 2), supporting the hypothesis that PikR1 monomethylates A2058.

The nature of the reactions catalyzed by PikR1 and PikR2 was corroborated by MS. A 50-nt-long 23S rRNA fragment encompassing A2058 was isolated from rRNA extracted from *E. coli* cells expressing PikR1 or PikR2 and subjected to RNaseA digestion and MALDI-TOF analysis. In the PikR1 sample, the peak corresponding to the unmodified RNA fragment $GGA_{2058}AAGAC$ (*m/z* 2,675) was almost absent, and a new peak at *m/z* 2,689 appeared (Fig. 2*C*), indicating that a methyl group had been added. In the corresponding 23S rRNA fragment from the PikR2 sample, the unmethylated RNA peak was also absent and replaced by a peak at *m/z* 2,703 showing the addition of two methyl groups (Fig. 2*D*). Combined with the results of primer extension analysis, the MS data showed that PikR1 and PikR2 target the same rRNA nucleotide but generate two different products: PikR1 monomethylates A2058, whereas PikR2 dimethylates this nucleotide.

***pikR1* Is Constitutively Expressed in the Native Host, Whereas *pikR2* Is Activated only When Antibiotics Are Produced.** Why does a ketolide producer that is equipped with the A2058 dimethyltransferase gene *pikR2* also need the *pikR1* gene, which has a product that merely monomethylates the same nucleotide? To address this question, we examined the regulation of expression of *pikR1* and

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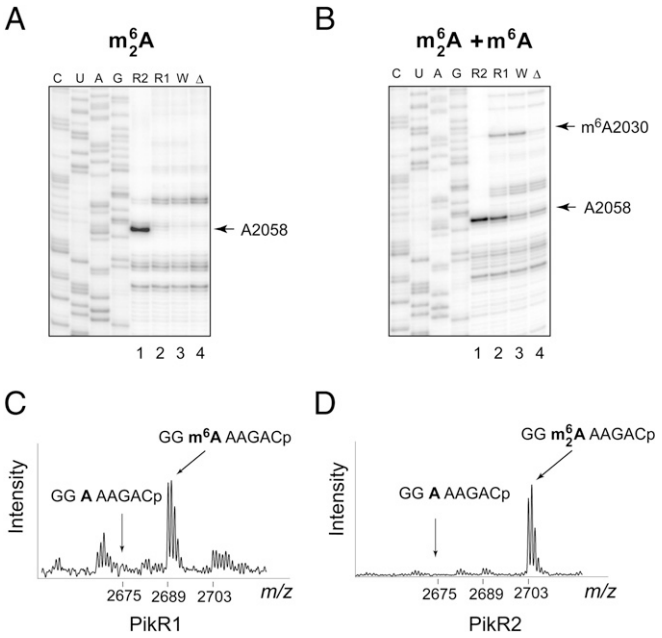

Fig. 2. PikR1 and PikR2 RNA methyltransferases target A2058 in the 23S rRNA. (*A*) Primer extension analysis of m_2^6A modification of rRNA extracted from WT *E. coli* cells (W; lane 3) or those constitutively expressing *pikR1* (R1; lane 2) and *pikR2* (R2; lane 1) genes. Sequencing lanes are marked C, U, A, G. Full gels are shown in Fig. S2*A*. (*B*) Primer extension analysis of the same samples as in *A* but carried out under conditions optimized for detection of m^6A modification (*Materials and Methods*). The *E. coli* Δ*rlmJ* mutant, which lacks the native m^6 modification of A2030 (33), was used as a control (Δ; lane 4). (*C* and *D*) MALDI-TOF analysis of the RNaseA-generated 23S rRNA fragment encompassing nucleotide A2058. rRNA samples were prepared from cells expressing (*C*) PikR1 or (*D*) PikR2.

pikR2 in *S. venezuelae*. When production of antibiotics was inactivated by deletion of either the *pikAI-pikAIV* genes (Δ*pikA*) or the *desI* gene (Δ*desI*) in the biosynthetic operon (leaving the *pikR1* and *pikR2* resistance genes intact) (Table S2), A2058 was found to be fully monomethylated (Fig. 3 and Fig. S2*B*), but there was essentially no dimethylation of this residue (Fig. 3 *A*, *D*, and *E*). This result indicated that *pikR1* is constitutively expressed, whereas *pikR2* remains inactive when no antibiotic is produced. In contrast, in the antibiotic-producing WT *S. venezuelae* ATCC 15439, a significant fraction (~30%) of A2058 was shown by primer extension to be converted to m_2^6A (Fig. 3*A* and Fig. S3), an observation confirmed by MS (Fig. 3*C*). Thus, *pikR2* is activated only when MTM and/or PKM are present in the cell. Because dimethylation of A2058 is known to reduce cell fitness (6), the inducible nature of *pikR2* is consistent with an evolutionary adaptation by the antibiotic-producing host to achieve secure protection against endogenously produced ketolides while maintaining a low fitness cost for this service.

Expression of *pikR2* Is Activated in the Natural Host by Clinically Relevant Ketolides. Induction of *pikR2* by ketolides of its native host suggested that the gene could also be activated by medically relevant ketolides. We tested whether preincubation with the clinical ketolide TEL could induce *pikR2* expression in *S. venezuelae*. Derivative strains containing only *pikR1* (DHS328) or only *pikR2* (DHS330) or lacking both genes (DHS332; designated here as R1, R2, or Δ, respectively) were prepared from the Δ*pikA* strain (DHS2001; designated here as WT*) (18). Without preincubation, the WT* and R1 cells showed an intermediate level of resistance to TEL [minimal inhibitory concentration (MIC) = 8 μg/mL], whereas R2 and Δ were highly sensitive (MIC = 0.25 μg/mL). Preincubation of the WT* and R2 strains with TEL concentrations of one-fourth MICs raised their respective resistance levels 8- and 64-fold, respectively (Table 1). The higher resistance of the WT* and R2 cells on exposure to TEL correlated with increased modification of nucleotide A2058 (Fig. S4). The microbiological and biochemical data thus show that, although constitutive expression of *pikR1* provides some protection from the clinical ketolide TEL, resistance is dramatically increased by drug-mediated activation of *pikR2*.

Molecular Mechanism of *pikR2* Induction. Macrolide resistance genes are activated by programmed ribosome stalling at the upstream leader ORFs (5, 19). Many of the resistance genes are induced exclusively or preferentially by cladinose-containing macrolides, whereas ketolides are poor inducers (10, 20). Indeed, ketolides owe their high activity against many clinical isolates to their low propensity for activation of inducible resistance genes.

We were interested in elucidating the molecular mechanism of ketolide-dependent induction of the *pikR2* gene. Examination of the *pikR2* upstream regions revealed the presence of a 16-codon ORF (*pikR2L*) 157 bp upstream of *pikR2* (Fig. 4*A*). The role of *pikR2L* in ketolide-mediated inducibility was investigated using a plasmid construct containing the *pikR2L* ORF, the intergenic region, and the first five codons of *pikR2* fused to the *lacZα* reporter gene (Fig. 4*B*). An *E. coli* Ptac promoter drives transcription of the reporter system. Induction of the *pikR2-lacZα* chimera was tested in *E. coli* by a disk diffusion assay (21). Natural and semisynthetic clinical ketolide antibiotics but notably, not the cladinose-containing macrolide ERY activated expression of the *pikR2L*-based reporter system (Fig. 4*B*). These data showed that ketolide-specific induction of *pikR2* occurs at the level of translation and is likely controlled by the upstream leader ORF.

The mechanism of translational induction was investigated in a cell-free transcription–translation system using a primer extension inhibition assay (toe printing) (22, 23) to test for ketolide- and macrolide-induced ribosome stalling on the *pikR2L* mRNA. Both MTM and PKM induced strong translation arrest at the Leu13 codon of *pikR2L*

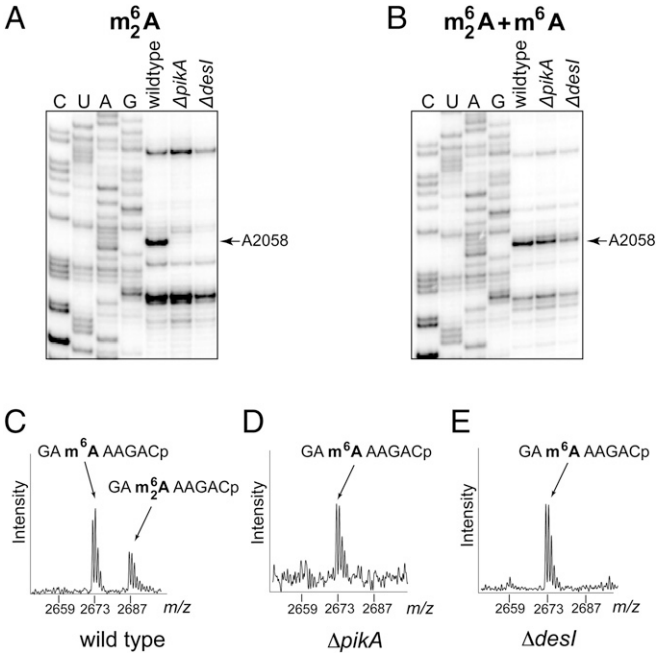

Fig. 3. Expression of *pikR2* in *S. venezuelae* is activated during antibiotic production. (*A* and *B*) Primer extension analysis of rRNA extracted from WT *S. venezuelae* ATCC 15439 carried under conditions specific for detection of (*A*) m_2^6A or (*B*) m_2^6A and m^6A modification. Full gels are shown in Fig. S2*B*. RNA was extracted from WT cells or mutants unable to produce active antibiotics because of deletion of the *pikAI-pikAIV* (Δ*pikA*) or *desI* (Δ*desI*) genes. Sequencing lanes in *A* and *B* are marked C, U, A, G. (*C–E*) MALDI-TOF analysis of 23S rRNA fragments from the WT or the Δ*pikA* and Δ*δεσI* KO mutants of *S. venezuelae*; the fragments were generated with RNaseA and encompass nucleotide A2058.

Table 1. MIC (micrograms per milliliter) of TEL or chloramphenicol for *S. venezuelae* Δ*pikA* strains containing different *pikR* resistance genes

	Antibiotics					
	TEL			Chloramphenicol		
	Preincubation			Preincubation		
Strain[†]	No	Yes[‡]	Fold change	No	Yes[‡]	Fold change
WT*	8	64	8	8	8	1
Δ	0.25	0.25	1	8	8	1
R1	8	16	2	8	8	1
R2	0.25	16	64	8	8	1

[†]The Δ*pikA* strain, which contained both *pikR1* and *pikR2* resistance genes but was unable to produce antibiotics (DHS2001), was designated WT* control. Formal names of the other strains are DHS332 (Δ), DHGS328 (R1), and DHS330 (R2).

[‡]Cells were grown for 8 h in LB supplemented with one-fourth MIC of TEL (0.0625 μg/mL for Δ and R2 strains or 2 μg/mL for WT* and R1 strains) before MIC testing.

(Fig. 4*C*, lanes 2 and 3). Unexpectedly, the clinical ketolide TEL was even more potent in arresting the ribosome at the Leu13 codon (Fig. 4*C*, lane 4). In contrast, consistent with the lack of in vivo induction of the *pikR2L* reporter system by ERY (Fig. 4*B*), this macrolide failed to induce ribosome stalling (Fig. 4*C*, lane 5). Taken together, these results suggest that *pikR2* has been evolutionarily optimized for specific activation by natural ketolide antibiotics and that semisynthetic clinical ketolides can serve as even more potent inducers.

Computational analysis shows that a stable stem-loop configuration of mRNA in the *pikR2L-pikR2* intergenic region may sequester the *pikR2* ribosome binding site (Fig. S5*A*). Ketolide-induced ribosome stalling at the Leu13 codon of *pikR2L* would destabilize the proximal mRNA stem and promote an alternative conformation, in which the initiation region of *pikR2* is accessible for translation (Fig. S5*B*).

Inducible Expression of *pikR2* Alone or Combined with *pikR1* Confers a High Level of Ketolide Resistance in a Clinically Relevant Host. After establishing that the natural resistance mechanisms conferred by *pikR1* and *pikR2* in the *Streptomyces* ketolide producer remain operational on transfer to *E. coli*, we extended the investigation to a heterologous Gram-positive model, because Gram-positive pathogens are the primary clinical targets of macrolides and ketolides. *Mycobacterium tuberculosis* is the etiological agent of tuberculosis and considered a possible target for ketolide therapy (24). We constructed strains of *Mycobacterium smegmatis*, a laboratory model of the pathogenic mycobacteria, which carried either the *pikR2* gene controlled by its regulatory region or the entire *pikR1-pikR2* cluster on a plasmid (Fig. 1*A*). The presence of *pikR2* alone elevated the resistance of *M. smegmatis* to clinical ketolides by 16- to 32-fold (Table 2). The combination of *pikR1* and *pikR2* conferred a much higher level of resistance (exceeding 1 mg/mL for TEL). Constitutive monomethylation of A2058 by PikR1 most likely facilitates continued ribosome activity and expression of *pikR2* on abrupt exposure of the cells to high concentrations of ketolides. When *M. smegmatis* cells with *pikR2* or *pikR1-pikR2* were preincubated with subinhibitory concentrations of TEL, resistance reached even higher levels, exceeding those of the control cells by several hundredfold (Table 2). Thus, the resistance genes originating in the producer of natural ketolides can render heterologous bacteria resistant to high concentrations of clinical ketolide antibiotics.

Discussion

In this paper, we present evidence that the genes *pikR1* and *pikR2* have been evolutionary optimized to confer resistance to ketolide antibiotics. These genes not only render *S. venezuelae* ATCC 15439 resistant to its endogenously produced ketolides but also, confer appreciable resistance to clinical ketolides. The resistance conferred by *pikR1* and *pikR2* can be transferred to heterologous Gram-negative and -positive hosts. We further show that the fitness cost of resistance is economized by the ketolide-inducible expression of *pikR2*. Taking these factors into account, acquisition of the *pikR1* and *pikR2* genes is expected to confer a marked selective advantage on bacteria undergoing repeated exposure to ketolides, which could promote the transfer of these genes to pathogens when ketolides become more widely used in a clinical setting.

Our studies provide insights into the important question of why *S. venezuelae*, the producer of the natural ketolides MTM and PKM, maintains two resistance genes with seemingly overlapping functions. The constitutively expressed PikR1 monomethylates 23S rRNA nucleotide A2058 located at the site of ketolide action, and this modification confers an intermediate level of resistance. Higher levels of resistance are attained with the inducible PikR2, which adds a second methyl group to the same nucleotide. The combined action of two differentially expressed genes ensures active protein synthesis in the ketolide-producing cells across a broad range of the inhibitors concentrations.

The inducibility of *pikR2* is significant for the operation of the resistance mechanism. Although dimethylation of A2058, at least in *Staphylococcus aureus*, is known to significantly reduce cell fitness (6), monomethylation of the same nucleotide seems to have little, if any, effect on *S. venezuelae* fitness (Fig. S6). Thus, with a minimal toll in fitness cost for the constitutive PikR1 monomethyltransferase, *S. venezuelae* easily tolerates low concentrations of MTM and/or PKM. However, when *S. venezuelae* augments its production of these drugs, activation of the more costly PikR2 enzyme is required, resulting in A2058 dimethylation and higher resistance levels. Producers of other natural antibiotics, which carry more than one resistance gene, may use a similar strategy for achieving the cost-effective protection from inhibitors (25).

In contrast to other inducible *erm* genes, which respond primarily to macrolides bearing a C3-cladinose, the regulation of *pikR2* has been evolutionary optimized for specific activation by ketolides (Fig. 4). Ketolide-specific *pikR2* induction is most likely controlled by programmed translation arrest within the *pikR2L* leader ORF. Cladinose-containing macrolides are prone to promoting early peptidyl-tRNA drop off occurring within the first 6–10 codons (26). Consistently, the sites of programmed macrolide-dependent translation arrest within the leader ORFs of the resistant genes are located within the ORF's first 10 codons, so that the drug-bound ribosome is able to reach the arrest site without prematurely dissociating from the mRNA (5, 19, 23, 27). Ribosomes translating *pikR2L* have to travel slightly farther and polymerize 13 amino acids before reaching the stalling codon. Because ketolides are less prone to promoting peptidyl-tRNA drop off (28), it is easier for ketolide-bound ribosomes than for ERY-bound ribosomes to polymerize the 13-aa-long nascent PikR2L chain. Indeed, the ERY-bound ribosome does not reach the stalling site (Fig. 4 and Fig. S5) and thus, fails to trigger *pikR2* expression. Ketolide-induced stalling in the *pikR2L* ORF occurs within the motif RLR, which represents one of the most problematic sequences for translation by ketolide-bound ribosomes (28, 29). We note that a short ORF is also found in front of the constitutively expressed

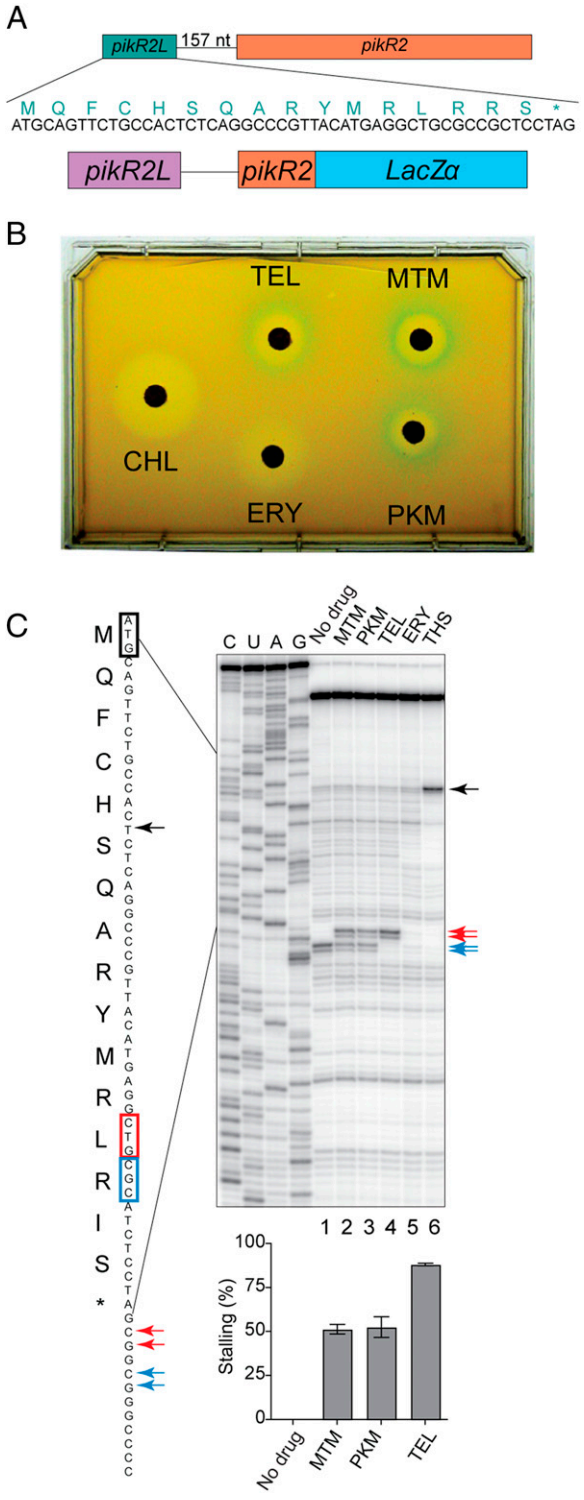

Fig. 4. The *pikR2* regulatory region controls the inducible expression of the *pikR2* resistance gene. (*A*) The putative leader ORF *pikR2L* precedes the *pikR2* resistance gene. The nucleotide sequence of the ORF and the amino acid sequence of the encoded leader peptide are shown. (*B*) Antibiotic disk diffusion assay reveals inducibility of *pikR2* in *E. coli*. In the reporter construct in *E. coli* cells, the *pikR2* regulatory region controls expression of the *lacZα* reporter. The antibiotic disks contained TEL, MTM, PKM, ERY, or chloramphenicol (CHL). The clear areas around the disks contain antibiotic concentrations that inhibited cell growth. Blue halos around the ketolide-containing disks indicate drug-dependent induction of the reporter. (*C*, *Upper*) Toe-printing analysis shows ketolide-induced ribosome stalling at the Leu13 codon of the *pikR2L* ORF. The band of the ribosomes stalled by the control antibiotic thiostrepton (THS) at the initiation codon is indicated by black arrows. The ribosomes arrested with the Leu13 codon in their P site are shown by the red arrows. The ribosomes that reached the *pikR2L* 13th codon but failed to arrest translation were captured at the next Arg14 codon (blue arrows) because of the depletion of Ile-tRNA from the translation reaction by the presence of the Ile-tRNA synthetase inhibitor, mupirocin. The stop codon or the ORF is indicated with an asterisk. (*C*, *Lower*) Stalling efficiency calculated from the ratios of the intensity of the bands representing ketolide-dependent arrest (codon 13) vs. read through (codon 14). Error bars indicate data spreads in two independent experiments.

pikR1 gene (Fig. S7*A*), although this ORF does not promote macrolide- or ketolide-dependent stalling (Fig. S7*B*) and thus, seems to play no role in the expression of *pikR1*.

The properties and regulation of the *pikR1* and *pikR2* genes are principally important for tolerating the broad range of inhibitor concentrations experienced by *S. venezuelae*. Constitutive monomethylation of A2058 by PikR1 renders ribosomes moderately resistant to ketolides, affording protection to *S. venezuelae* at the early stages of antibiotic production. Monomethylation of A2058 is a critical step toward acquisition of a high level of resistance, facilitating efficient translation of *pikR2*, despite the presence of a ketolide inhibitor. Indeed, on abrupt exposure of the *S. venezuelae pikR1*-null mutant (Δ*pikR1*) to TEL, the lone *pikR2* gene was unable to provide any significant protection (Table 1), but when both *pikR* genes were present and cells were preincubated with low ketolide concentrations (mimicking the onset of antibiotic production), a high level of resistance was achieved. In the absence of *pikR1*, the resistance of *S. venezuelae* to TEL afforded by *pikR2* alone, even on preincubation with the antibiotic, was no higher than that provided by the constitutively expressed monomethylase (Table 1). This intermediate level of resistance may be caused by either dimethylation of only a small fraction of ribosomes or mixed mono- and dimethylations, because Erm dimethyltransferases are known to add two methyl groups to A2058 in two consecutive reactions (30).

The synergy between the *pikR1* and *pikR2* genes was observed to an even greater extent in *M. smegmatis*, which was used to model the expression of the *pikR* genes in clinical pathogens. Here, the *pikR2* gene alone conferred considerable resistance to clinical ketolides. However, it was the simultaneous presence of both *pikR1* and *pikR2* genes that provided the highest resistance (Table 2). The resistance to TEL afforded by *pikR1* and *pikR2* in *M. smegmatis* was significantly higher than in *S. venezuelae*. Factors that could contribute to this effect include the increased dosage of the resistance genes introduced into mycobacteria on a plasmid, the slower subunit assembly resulting in a higher proportion of methylated rRNA, or the lower affinity of the antibiotic for modified mycobacterial ribosomes.

The inducibility of *pikR2*, which lowers the fitness cost of the two-gene resistance mechanism, may facilitate not only its acquisition by a new host through horizontal gene transfer but also, its maintenance on discontinuation of the antibiotic treatment. Horizontal transfer of the *pikR1*/*pikR2* gene pair may be further facilitated by their close physical proximity in the *S. venezuelae* chromosome (Fig. 1*A*). Despite the high GC content of *pikR1* and *pikR2*, our results strongly suggest that their transfer into at least some clinical strains would render the strains highly resistant to ketolide therapy. These findings, thus, provide additional justification for renewed drug discovery efforts to identify novel antibiotics capable of overcoming protection rendered by natural resistance genes.

Materials and Methods

For isolation of the total RNA from *S. venezuelae*, cells were grown overnight in SGGP media, then diluted 1:100 in SCM media (31), and grown for 5 d at 30 °C with constant shaking. Cells were pelleted from 5-mL cultures, resuspended in 1 mL Tris-EDTA (TE) buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA) containing 5 mg/mL lysozyme, incubated for 10 min at room temperature, and then, shaken for 10 min in the Minibead Breaker (Biospec Products) with 50 μL glass beads (particle size ≤106 μm; Sigma-Aldrich). Total RNA was isolated using the RNeasy Maxi Kit (Qiagen).

The RNeasy Plus Mini Kit was used to prepare total RNA from exponentially growing *E. coli* following the standard manufacturer's protocol. Primer extension analysis under the standard conditions suitable for detection of adenine N6 dimethylation was carried out as previously described (21). In experiments where

Table 2. MIC (micrograms per milliliter) of clinically relevant ketolides for *M. smegmatis* harboring the *pikR2* gene alone or the combination of *pikR1* and *pikR2* genes

	TEL		SOL		CET	
	Preincubation		Preincubation		Preincubation	
Plasmid	No	Yes*	No	Yes*	No	Yes*
pMIP12 (vector)	2	2	1	1	1	2
pMR2	64	2,048	16	1,024	32	512
pMR1R2	1,024	2,048	256	1,024	256	512

Antibiotics used were TEL, solithromycin (SOL), and cethromycin (CET). The *M. smegmatis* strain used in MIC testing was a derivative of the strain mc^2155, in which the endogenous macrolide resistance gene *erm38* was inactivated by allelic exchange (34).

*Cells were grown for 72 h in the medium supplemented with one-eighth MIC of TEL (0.25 μg/mL for cells transformed with the empty vector, 8 μg/mL for cells transformed with pMR2, or 128 μg/mL for cells transformed with pMR1R2) before MIC testing.

detection of N6 monomethylation was required, the concentration of dTTP was reduced from 1 to 0.01 mM with extension for 15 min at 37 °C instead of the standard 30 min at 42 °C. Primers L2180, L2563, and L2667 (Table S3) were used to examine domain V of 23S rRNA of *E. coli*. Primer L2405-R was used to check the modification status of A2058 in 23S rRNA of *S. venezuelae*.

Toe printing was carried out as described previously (23). The status of rRNA modification in *E. coli* and *S. venezuelae* was analyzed using the approach described in ref. 32.

Other experimental details are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Brian Murphy, Skylar Carlson, and Xiaomei Wei for help in analysis of pikromycin (PKM) and methymycin (MTM); Dorota Klepacki for the *E. coli* Δ*acrA* strain; and Prabha Fernandes (Cempra, Inc.) for providing clinical ketolides. M.M.A. was supported by the King Saud University Scholarship for Graduate Studies. D.A.H. was supported by a University of Michigan Rackham Merit Predoctoral Fellowship. This work was supported by National Institutes of Health Grants R01 GM106386 (to N.V.-L. and A.S.M.) and R01 GM076477 (to D.H.S.), Danish Research Agency Forskningsrådet for Natur og Univers (FNU)-Rammebevilling Grant 10-084554 (to S.D.), and the Hans W. Vahlteich Professorship (D.H.S.).

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