Two Distinct Major Facilitator Superfamily Drug Efflux Pumps Mediate Chloramphenicol Resistance in *Streptomyces coelicolor*[▽]

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Chloramphenicol, florfenicol, and thiamphenicol are used as antibacterial drugs in clinical and veterinary medicine. Two efflux pumps of the major facilitator superfamily encoded by the *cmlR1* and *cmlR2* genes mediate resistance to these antibiotics in *Streptomyces coelicolor*, a close relative of *Mycobacterium tuberculosis*. The transcription of both genes was observed by reverse transcription-PCR. Disruption of *cmlR1* decreased the chloramphenicol MIC 1.6-fold, while disruption of *cmlR2* lowered the MIC 16-fold. The chloramphenicol MIC of wild-type *S. coelicolor* decreased fourfold and eightfold in the presence of reserpine and Phe-Arg- β -naphthylamide, respectively. These compounds are known to potentiate the activity of some antibacterial drugs via efflux pump inhibition. While reserpine is known to potentiate drug activity against gram-positive bacteria, this is the first time that Phe-Arg- β -naphthylamide has been shown to potentiate drug activity against a gram-positive bacterium.

Membrane-bound efflux pumps commonly underlie drug resistance in pathogenic bacteria (21, 26, 33). These pumps function by actively expelling drugs from the cytosol of bacterial cells, reducing the effective intracellular drug concentration. There are five major families of efflux pumps in bacteria: the ATP binding cassette family, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion family, the resistance nodulation division family, and the small multidrug resistance family (33). Some efflux pumps exhibit high specificity for certain antimicrobial agents, while others act upon drugs from unrelated structural classes and thereby confer multidrug resistance (33). In both gram-positive and gram-negative bacteria, genes encoding efflux pumps are found on the chromosome and on plasmids (33). Multidrug resistance in human pathogens is often correlated with the overexpression of efflux pump genes (18, 34).

Efflux is a nondestructive mechanism of antibiotic resistance commonly observed in antibiotic-producing bacteria that provides self-resistance without compromising the biological activity of the biosynthesized antibiotics (9). Given that antibiotic-producing organisms are a reservoir of resistance genes (23), they are a likely source of the drug efflux pump genes found in pathogenic bacteria. *Streptomyces* bacteria produce two-thirds of the clinically used antibiotics and often have an efflux pump gene associated with self-resistance (1, 17, 31). In some cases, these gram-positive, soil-dwelling bacteria have efflux pumps that confer resistance to multiple antibiotics. For instance, the *ptr* gene in *Streptomyces pristinaespiralis* confers resistance to the pristinamycins and rifampin (rifampicin) (3). Likewise, an ATP binding cassette family transporter gene in *Streptomyces rochei* F20 confers resistance to macrolides (i.e., oleandomycin, erythromycin, and spiramycin), tetracycline, and doxorubicin (11). Although most *Streptomyces* bacteria are not pathogenic,

they are of clinical interest because they are close relatives of pathogenic mycobacteria and are considered to be a reservoir of resistance genes (1, 9).

In this study, we examined efflux pump-mediated drug resistance in *Streptomyces coelicolor*, the model organism of the *Streptomyces* genus (1). *S. coelicolor* has an extensive array of efflux pump genes proposed to confer high-level resistance to a range of drug-related compounds that it does not produce, including macrolides, streptogramins, fosmidomycin, and chloramphenicol (1; <http://streptomyces.org.uk>; <http://www.membranetransport.org/transporter2.php?oOID=scoe1>). Presumably, *S. coelicolor* acquired these genes by horizontal transfer. We have established that two of the MFS efflux pump genes in *S. coelicolor* confer resistance to chloramphenicol, thiamphenicol, and florfenicol. These genes, *cmlR1* and *cmlR2*, have low levels of homology to one another, yet each gene independently contributes to the chloramphenicol resistance of *S. coelicolor*. We also found that two known efflux pump inhibitors, reserpine and Phe-Arg- β -naphthylamide, significantly increased the susceptibility of *S. coelicolor* to chloramphenicol. While reserpine is known to potentiate drug activity against gram-positive bacteria, this is the first time that Phe-Arg- β -naphthylamide has been shown to potentiate the activity of a drug against a gram-positive bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *S. coelicolor* strains used in this work are provided in Table 1. The *S. coelicolor* strains were grown at 30°C on mannitol soy flour medium (SFM), Difco nutrient agar medium, yeast extract-malt extract medium, or minimal liquid medium (NMMP) (16). SFM was used for conjugations between *S. coelicolor* and *Escherichia coli* and for generating spore stocks. *Escherichia coli* strains DH5 α and ET12567/pUZ8002 were grown on Luria-Bertani medium at 37°C for routine subcloning (29), and *E. coli* strain BW25113/pIJ790 was grown on Luria-Bertani medium at 30°C when selection for pIJ790 was maintained. For transcriptional analysis, *S. coelicolor* was grown in NMMP. For the selection of *E. coli*, ampicillin, apramycin, chloramphenicol, hygromycin, and kanamycin were employed at 100, 50, 25, 80, and 50 μ g/ml, respectively. Nalidixic acid was used at 20 μ g/ml to counterselect *E. coli* in conjugations with *S. coelicolor*. Apramycin and hygromycin were used at 50 μ g/ml for the selection of *S. coelicolor* exconjugants.

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TABLE 1. *S. coelicolor* strains and plasmids used in this study

Strain or plasmid	Genotype or description ^a	Reference or source
<i>S. coelicolor</i> strains		
M600	Prototroph SCP1 [−] SCP 2 [−]	16
B754	M600 <i>cmlR1::apr</i>	This study
B755	M600 <i>cmlR1::apr</i> pMS81- <i>cmlR1</i> locus	This study
B756	M600 <i>cmlR2::apr</i>	This study
B757	M600 <i>cmlR2::apr</i> pMS81- <i>cmlR2</i> locus	This study
B758	M600 <i>cmlR2::apr</i> pIJ10257- <i>cmlR1</i> ORF	This study
B759	M600 <i>cmlR2::apr</i> pIJ10257- <i>cmlR2</i> ORF	This study
Plasmids		
pMS81	<i>oriT</i> , ΦBT1 <i>attB-int</i> , Hyg ^r , derivative of pSET152	12
pIJ10257	<i>oriT</i> , ΦBT1 <i>attB-int</i> , Hyg ^r , <i>ermEp*</i> , derivative of pMS81	14
pJS331	<i>cmlR1</i> locus cloned into pMS81 for complementation	This study
pJS332	<i>cmlR2</i> locus cloned into pMS81 for complementation	This study
pJS333	<i>cmlR1</i> ORF cloned into pIJ10257 for overexpression	This study
pJS334	<i>cmlR2</i> ORF cloned into pIJ10257 for overexpression	This study

^a Hyg^r, hygromycin resistance; *apr*, the apramycin resistance cassette.

Plasmids and primers. Standard cloning procedures (29) were employed to generate the plasmids listed in Table 1. The site-specific integrating vector pMS81 (12) was used for genetic complementation (32). pIJ10257, derived from pMS81, was the vector used for the overexpression of the efflux pump genes in *S. coelicolor* (14). DNA sequencing was performed by Davis Sequencing (Davis, CA). All primers used in the work were synthesized by Invitrogen. PCR was performed with *Taq* (Invitrogen) and *Pfu* (Stratagene, Agilent Technologies). All PCRs were performed with 5% (vol/vol) dimethyl sulfoxide (16).

Construction of *cmlR1*- and *cmlR2*-null mutants. PCR-targeted mutagenesis was used to replace the *S. coelicolor* SCO7526 (*cmlR1*) and SCO7662 (*cmlR2*) genes with an apramycin resistance marker, *apr* (13). The requisite PCR products were amplified from the apramycin resistance cassette of pIJ773 with primers 5 to 8 (Table 2). The PCR products were introduced into *E. coli* BW25113/pIJ790

harboring either cosmid St8G12 (*cmlR1*) or cosmid St10F4 (*cmlR2*) and expressing bacteriophage λ RED recombinase. The resultant recombinant cosmids, St8G12 Δ*cmlR1::apr* and St10F4 Δ*cmlR2::apr*, were introduced into *E. coli* strain ET12567/pUZ8002 and then into wild-type strain *S. coelicolor* M600 (16) by way of conjugation, as described previously (15). Exconjugants lacking the *cmlR1* and *cmlR2* genes were identified by selection for apramycin resistance and kanamycin sensitivity. Gene replacements in the *cmlR1*-null strain (strain B754) and in the *cmlR2*-null strain (strain B756) were confirmed by PCR analysis of genomic DNA isolated from the null mutants with primers 9 to 12 (Table 2).

Complementation of *cmlR1*- and *cmlR2*-null mutants. A 1,724-bp DNA fragment containing *cmlR1* and its promoter region was excised from *S. coelicolor* cosmid St8G12 by restriction digestion with *Sfi*I and *Sma*II. This fragment contained the *cmlR1* open reading frame (ORF) with 430 upstream base pairs. The excised fragment was treated with DNA polymerase I large (Klenow) fragment (New England Biolabs), according to the manufacturer's protocol, and ligated into the *Sma*I site of pBluescript II KS+. The fragment was then excised from pBluescript II KS+ with *Spe*I and *Eco*RV and ligated into pMS81 pretreated with the same enzymes to yield pJS331. pJS331 was introduced by transformation into nonmethylating *E. coli* strain ET12567/pUZ8002 and then into the *S. coelicolor* *cmlR1*-null mutant via conjugation.

A 1,882-bp DNA fragment containing *cmlR2* and its promoter region was excised from *S. coelicolor* cosmid St10F4 by restriction digestion with *Sph*I. This fragment contained the *cmlR2* ORF with 579 upstream base pairs. The excised *Sph*I fragment was treated with DNA polymerase I large (Klenow) fragment (New England Biolabs), according to the manufacturer's protocol, and ligated into the *Sma*I site of pBluescript II KS+. The fragment was then excised from pBluescript II KS+ and ligated into pMS81 to yield pJS332, which was introduced into the *S. coelicolor* *cmlR2*-null mutant as described above.

Overexpression of *cmlR1* and *cmlR2* genes. The *cmlR1* and *cmlR2* ORFs were amplified from wild-type *S. coelicolor* genomic DNA with primers 1 to 4 (Table 2). The PCR products were cloned into pBluescript II KS+ and sequenced to confirm their identities. The genes were transferred into the integrative, constitutive expression vector pIJ10257 (14), yielding pJS333 (*cmlR1*) and pJS334 (*cmlR2*). The resultant plasmids were introduced into *E. coli* strain ET12567/pUZ8002 and then into the chloramphenicol-sensitive *S. coelicolor* *cmlR2*-null strain (strain B756) by way of conjugation, as described previously (15). Exconjugants with either pJS333 (strain B758) or pJS334 (strain B759) were identified by selection for hygromycin resistance.

Antimicrobial agents. Chloramphenicol, florfenicol, thiamphenicol, reserpine, and Phe-Arg-β-naphthylamide were all purchased from Sigma-Aldrich.

MIC assays. All MIC assays were performed on Difco nutrient agar medium supplemented with the indicated concentrations of each antimicrobial agent. Growth was assessed after incubation at 30°C for 48 h.

TABLE 2. Primers used in this study

Entry	Primer name	Application or function	Sequence
1	<i>cmlR1</i> ORF F	Cloning of the <i>cmlR1</i> ORF	5'-CATATGCCGCTCGCCGTCTAC-3' ^a
2	<i>cmlR1</i> ORF R	Cloning of the <i>cmlR1</i> ORF	5'-CTCGAGTCACACCCGCTGGGCC-3' ^b
3	<i>cmlR2</i> ORF F	Cloning of the <i>cmlR2</i> ORF	5'-CATATGCCCTCTCCGCTGTAC-3' ^a
4	<i>cmlR2</i> ORF R	Cloning of the <i>cmlR2</i> ORF	5'-CTCGAGTCACCGGGTCCGCTC-3' ^b
5	<i>cmlR1</i> KO F	PCR-targeted mutagenesis of <i>cmlR1</i>	5'-ATGCCCGACTCTTCCCCACGGTCCACGGCCGGACGC ATGACTAGTATTCCGGGGATCCGTCGACC-3'
6	<i>cmlR1</i> KO R	PCR-targeted mutagenesis of <i>cmlR1</i>	5'-CCCGGGTCACACCCGCTGGGCCCGCTCCAGGCTCG CGGTAAGTGTAGGCTGGAGCTGCTTC-3'
7	<i>cmlR2</i> KO F	PCR-targeted mutagenesis of <i>cmlR2</i>	5'-CCTTTCTCGTGCTTCTCCTCGCCTCGGAGCCACTCGA TGACTAGTATTCCGGGGATCCGTCGACC-3'
8	<i>cmlR2</i> KO R	PCR-targeted mutagenesis of <i>cmlR2</i>	5'-GGGACACGACGTGGTACGCCAGTCGGGTGGCGGT TTCAACTAGTTGTAGGCTGGAGCTGCTTC-3'
9	<i>cmlR1</i> KD F	Confirmation of <i>cmlR1</i> replacement with <i>apr</i>	5'-GGCATGCCCGACTCTTC-3'
10	<i>cmlR1</i> KD R	Confirmation of <i>cmlR1</i> replacement with <i>apr</i>	5'-CTGGCCGACCGCTGTG-3'
11	<i>cmlR2</i> KD F	Confirmation of <i>cmlR2</i> replacement with <i>apr</i>	5'-GCCGTGGTGACGAGATG-3'
12	<i>cmlR2</i> KD R	Confirmation of <i>cmlR2</i> replacement with <i>apr</i>	5'-GTGCGCGGGACACGAC-3'
13	<i>cmlR1</i> RT F	Detection of the <i>cmlR1</i> transcript via RT-PCR	5'-GTCTACATCCTCGGCTGTC-3'
14	<i>cmlR1</i> RT R	Detection of the <i>cmlR1</i> transcript via RT-PCR	5'-GGGGATGAGGGTCACCAC-3'
15	<i>cmlR2</i> RT F	Detection of the <i>cmlR2</i> transcript via RT-PCR	5'-CTCTTCGCTGTACCTGCTC-3'
16	<i>cmlR2</i> RT R	Detection of the <i>cmlR2</i> transcript via RT-PCR	5'-CATGTGCCGAGGAGTGAC-3'

^a The engineered *Nde*I site is underlined.

^b The engineered *Xho*I site is underlined.

TABLE 3. MIC analysis associates *S. coelicolor* genes *cmlR1* and *cmlR2* with drug efflux

Compound	Wild-type strain MIC (μg/ml)	<i>cmlR1::apr</i>		<i>cmlR2::apr</i>	
		MIC (μg/ml)	Fold MIC reduction ^a	MIC (μg/ml)	Fold MIC reduction
Chloramphenicol	80	50	1.6	5	16
Florfenicol	30	17	1.8	10	3
Thiamphenicol	>500	>500		50	>10

^a The MIC of each mutant strain was compared to the MIC of the wild-type strain to obtain the MIC reductions.

RNA isolation and RT-PCR. Transcriptional analyses of the *S. coelicolor* SCO7526 and SCO7662 genes were performed as described previously (32). Two NMMP cultures were inoculated with 2×10^9 *S. coelicolor* spores to test for transcription in the presence and the absence of chloramphenicol. One culture was grown for 21 h prior to treatment with 50 μg/ml chloramphenicol for 3 h. The cells were then washed once with 10.3% (wt/vol) aqueous sucrose; resuspended in lysis buffer consisting of 50 mM Tris-HCl, pH 8.0–1 mM EDTA supplemented with 10 mg/ml lysozyme (Sigma-Aldrich); and incubated for 15 min at 30°C. Total RNA was then isolated with an RNeasy minikit (Qiagen), according to the manufacturer's protocol. The concentration of each purified, DNA-free total RNA isolate was measured with a NanoDrop ND-1000 spectrophotometer. An equal quantity of total RNA (1.8 μg) was employed in all reverse transcription-PCRs (RT-PCRs). RT-PCR was performed with a OneStep RT-PCR kit (Qiagen), according to the manufacturer's protocol for transcripts with high GC contents (by the use of Q solution). Primers 13 to 16 (Table 2) were used for RT-PCR analyses. The PCR program used for the detection of both transcripts was 50°C for 30 min; 95°C for 15 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s; and a final elongation at 72°C for 10 min. No signals were detected in control experiments with *Pfu* polymerase, indicating that the RT-PCR signals correspond to the amplification of transcripts.

RESULTS

MFS efflux pumps encoded by the *cmlR1* and *cmlR2* genes provide drug resistance in *S. coelicolor*. *S. coelicolor* is highly resistant to chloramphenicol (MIC, 80 μg/ml) (Table 3). Bioinformatic analysis of the *S. coelicolor* genome sequence revealed two putative MFS chloramphenicol efflux pump genes, *cmlR1* (SCO7526) and *cmlR2* (SCO7662) (1). These genes are separated by more than 100 kb on the chromosome and encode proteins that are 40% identical in amino acid sequence. The products of *cmlR1* and *cmlR2* are related to the MFS chloramphenicol exporters CmlA in *E. coli* and FloR in *Salmonella enterica* (4, 5). Interestingly, *Mycobacterium tuberculosis* has an uncharacterized MFS protein that is also homologous to the products of *cmlR1* and *cmlR2* (7).

Initially, we used RT-PCR to assess the transcriptional activity of these genes. Both *cmlR1* and *cmlR2* were constitutively transcribed; the transcripts were detected in both the presence and the absence of chloramphenicol (Fig. 1). To validate their functions and determine their relative contributions to chlor-

FIG. 1. Detection of the *cmlR1* and *cmlR2* transcripts via RT-PCR. The transcripts were detected in *S. coelicolor* shaken liquid cultures grown in the presence and the absence of chloramphenicol.

TABLE 4. Effect of reserpine on chloramphenicol susceptibility

<i>S. coelicolor</i> strain	MIC (μg/ml) in the presence of reserpine at ^a :				
	None	10 μg/ml	25 μg/ml	50 μg/ml	75 μg/ml
Wild type	80	60 (1.3)	30 (2.7)	20 (4.0)	20 (4.0)
<i>cmlR1::apr</i>	50	20 (2.5)	20 (2.5)	15 (3.3)	15 (3.3)
<i>cmlR2::apr</i>	5	4 (1.3)	2.5 (2.0)	2.5 (2.0)	2.5 (2.0)

^a Numbers in parentheses indicate the MIC reductions (fold) compared to the MICs of strains grown in the absence of reserpine.

amphenicol resistance, we used a PCR-targeting procedure to replace them with an apramycin resistance marker in wild-type strain *S. coelicolor* M600 (13). The gene replacements were confirmed by PCR analysis of genomic DNA isolated from the null strains. Both null strains, *S. coelicolor* B754 *cmlR1::apr* and *S. coelicolor* B756 *cmlR2::apr*, were grown on Difco nutrient agar to assess their chloramphenicol susceptibilities. The chloramphenicol MIC of the strain lacking *cmlR1* (50 μg/ml) was 1.6-fold lower than that of the wild-type strain, and the MIC of the strain lacking *cmlR2* (5 μg/ml) was 16-fold lower (Table 3). These results indicate that *cmlR2* encodes the major chloramphenicol resistance determinant in *S. coelicolor*. In both null strains, chloramphenicol resistance was restored by genetic complementation in *trans* (strains B755 and B757 in Table 1). To further validate the conclusion that *cmlR1* is a minor contributor to chloramphenicol resistance, we generated strains B758 and B759 in which either the *cmlR1* gene or the *cmlR2* gene, respectively, was overexpressed in the chloramphenicol-sensitive *cmlR2*-null strain (Table 1). The overexpression of the *cmlR1* gene increased the chloramphenicol MIC from 5 μg/ml to 10 μg/ml (a 2-fold increase), while the overexpression of the *cmlR2* gene increased the MIC from 5 μg/ml to 70 μg/ml (a 14-fold increase).

The null strains were also tested for their susceptibilities to florfenicol and thiamphenicol. The florfenicol MIC of the wild-type *S. coelicolor* strain was 30 μg/ml (Table 3). Strains lacking *cmlR1* and *cmlR2* exhibited 1.8-fold and 3-fold florfenicol MIC reductions, respectively (Table 3). In contrast, the thiamphenicol MICs of wild-type *S. coelicolor* and the strain lacking *cmlR1* were both found to be greater than 500 μg/ml (Table 3). The thiamphenicol MIC of the strain lacking *cmlR2* was reduced 10-fold compared to that of wild-type *S. coelicolor* (Table 3). These results indicate that *cmlR2*, and not *cmlR1*, is a thiamphenicol resistance determinant. Thiamphenicol and florfenicol resistance was restored to the null strains by genetic complementation in *trans*.

Reserpine and Phe-Arg-β-naphthylamide markedly increase the susceptibility of *S. coelicolor* to chloramphenicol. Reserpine is a plant alkaloid known to potentiate the activities of fluoroquinolones and tetracycline in gram-positive bacteria by inhibiting multidrug efflux pumps (6, 8, 24, 30). In light of the ability of reserpine to potentiate drug activity in gram-positive bacteria, we assessed the chloramphenicol susceptibilities of wild-type *S. coelicolor* and the *cmlR1*-null and *cmlR2*-null strains over a range of reserpine concentrations (Table 4). Wild-type *S. coelicolor* was rendered more susceptible to chloramphenicol at all reserpine concentrations tested, but the most significant potentiation of chloramphenicol activity (i.e., fourfold) was observed at reserpine concentrations at or above 50 μg/ml

TABLE 5. Effect of Phe-Arg- β -naphthylamide on chloramphenicol susceptibility

<i>S. coelicolor</i> strain	MIC (μ g/ml) in the presence of PABN at ^a :				
	None	10 μ g/ml	25 μ g/ml	50 μ g/ml	75 μ g/ml
Wild type	80	30 (2.7)	25 (3.2)	20 (4.0)	10 (8.0)
<i>cmlR1::apr</i>	50	20 (2.5)	15 (3.3)	15 (3.3)	10 (5.0)
<i>cmlR2::apr</i>	5	3 (1.7)	3 (1.7)	3 (1.7)	3 (1.7)

^a Numbers in parentheses indicate the MIC reductions (fold) compared to the MICs of strains grown in the absence of Phe-Arg- β -naphthylamide (PABN).

(Table 4). In contrast, the activity of chloramphenicol against the *cmlR1*- and *cmlR2*-null strains was potentiated less than fourfold at all reserpine concentrations tested (Table 4). Clearly, potentiation was dependent on both the dose of reserpine and the genetic background of the strain being tested. In control experiments, reserpine alone had no apparent toxicity to *S. coelicolor*. In fact, the reserpine MIC of *S. coelicolor* could not be determined at concentrations below 250 μ g/ml.

Phe-Arg- β -naphthylamide is a synthetic C-capped dipeptide known to potentiate drug activity in gram-negative bacteria via inhibition of the resistance nodulation division family of drug efflux pumps (2, 19–21, 25, 27). It has been reported that *E. coli* strains harboring multidrug efflux pumps are more susceptible to chloramphenicol in the presence of Phe-Arg- β -naphthylamide (28). On the basis of those observations, we sought to determine if this compound would increase the chloramphenicol susceptibilities of wild-type *S. coelicolor* and the *cmlR1*- and *cmlR2*-null strains (Table 5). The chloramphenicol susceptibility of wild-type *S. coelicolor* increased in proportion to the concentration of Phe-Arg- β -naphthylamide, with an eightfold potentiation observed at 75 μ g/ml (Table 5). In the presence of Phe-Arg- β -naphthylamide, chloramphenicol activity was potentiated to a lesser degree in the *cmlR1*- and *cmlR2*-null strains than in the wild-type strain (Table 5). As was the case for reserpine, the potentiation of chloramphenicol activity by Phe-Arg- β -naphthylamide was dependent on both the compound dose and the genetic background of the strain being tested. In control experiments, Phe-Arg- β -naphthylamide alone had no apparent toxicity to *S. coelicolor*. The Phe-Arg- β -naphthylamide MIC of *S. coelicolor* was 200 μ g/ml.

DISCUSSION

Bacterial drug efflux pumps have the potential to critically compromise the efficacies of many antibacterial drugs in use today (33). In many cases, the genes encoding efflux pumps are found on plasmids that are easily transferred from one bacterium to another. Certain bacteria have multiple genes encoding efflux pumps that mediate multidrug resistance. *S. coelicolor* is a multidrug-resistant bacterium (9, 14, 16, 32) with a large number of putative drug efflux pumps (1). The mechanism of chloramphenicol resistance described here is particularly interesting because *S. coelicolor* has two distinct MFS efflux pumps that confer chloramphenicol resistance, yet it does not produce the antibiotic. Transcriptional analyses clearly indicate that both genes are constitutively transcribed. Using reverse genetic analysis, we demonstrated that the *cmlR2* gene is the major contributor to chloramphenicol

resistance. A close ortholog of *cmlR2* has been observed in *Streptomyces lividans* (10). It is intriguing that *S. coelicolor* has retained and simultaneously transcribes two antibiotic resistance genes with redundant functions.

We also found that *S. coelicolor* was sensitized to chloramphenicol in the presence of reserpine and Phe-Arg- β -naphthylamide, two well-known efflux pump inhibitors from distinct structural classes (21, 22). Interestingly, Phe-Arg- β -naphthylamide has never been reported to enhance the antibacterial susceptibility of a gram-positive bacterium. It is also noteworthy that the potentiation of chloramphenicol activity by reserpine and Phe-Arg- β -naphthylamide was diminished in *S. coelicolor* strains lacking either the *cmlR1* or the *cmlR2* gene. This observation suggests that inhibition of CmlR1 and CmlR2 may be the underlying mechanism of potentiation. In any case, our findings provide further evidence that efflux pump inhibitors can be useful against multidrug-resistant, gram-positive bacteria (8).

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