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Mutation in the *Bacillus subtilis* RNA Polymerase β′ Subunit Confers Resistance to Lipiarmycin

Lipiarmycin (Lprm) (8), a macrocyclic antibiotic also known as tiacumicin (2), is currently under development under the name of OPT-80 (Optimer Pharmaceuticals, Inc., San Diego, Calif.) as a narrow-spectrum antibacterial agent to treat *Clostridium difficile*-associated diarrhea (1, 7).

Lprm is a transcription inhibitor, but unlike rifampin and streptolydigin, it preferentially inhibits holoenzyme transcription at a much greater rate than it inhibits transcription of the core enzyme (5, 6). Genetic mapping experiments in *Bacillus subtilis* indicate that mutants are located between loci determining rifampin resistance and streptolydigin resistance (6). To precisely identify the positions of the mutations, we selected lipiarmycin-resistant colonies and sequenced the domains of the RNA polymerase located between these two loci.

Lprm was produced and purified by fermentation of *Actinoplanes deccanensis* (DSMZ 43806) by the method of Talpaert et al. (8). Mutant strains were isolated as spontaneous variants of *Bacillus subtilis* CIP 52.62 that were able to form colonies on nutrient agar plates containing Lprm (40 μg/liter). At this concentration the frequency of resistant colonies was less than 10^{-7}. Among the 10 resistant colonies selected, 8 exhibited an increased MIC for several classes of antibiotics and were likely to be permeability mutants (Table 1). Two mutants (mut1 and mut2) were highly resistant to Lprm.

To correlate the resistance phenotypes with the activity of Lprm on the RNA polymerase, we tested enzymes from the wild-type and mutant colonies in an in vitro transcription assay by the method of Sonenshein et al. (6). The parent cells (CIP 52.62) and mutant cells specifically resistant to Lprm (mut1 and mut2) showed the same pattern of sensitivity to rifampin. In contrast, the transcription activity of the wild-type cells was more strongly affected by the addition of Lprm than was the activity of either mut1 or mut2. This confirmed the link between resistance to Lprm resistance and transcription. mut3 to mut10 strains were equally sensitive to rifampin and Lprm, suggesting that the resistance was not due to a mutation in the polymerase.

After purification of the genomic DNA, we PCR amplified and sequenced the regions located between the loci determining rifampin and streptolydigin resistance. The following primers were used: 5′-^{1285}CGTGTGGTTCGTGAGAGAATGT^{1306}-3′, 5′-^{3257}TAAGCTTCAAGTGCCCAAACCT^{3236}-3′, and 5′-^{2524}CTTGTTGGTAAAGTAACGCCTA^{2545}-3′ for *rpoB* and 5′-^{1163}CGTTTCGCACTCTTAATGTTGTG^{1141}-3′, 5′-^{593}CACAAGGACAACGCCGTAC^{611}-3′, and 5′-^{2804}GTTAACTGTGTACCAGGCTCACC^{2782}-3′ for *rpoC*. A point mutation resulting in the substitution of the R326 of *rpoC* by L (CG[T/C]TT) was found in mut1 and mut2 in a highly conserved region; no mutation was detected in mut3 to mut10. When the PCR product harboring the R326L mutation was transfected into *B. subtilis* CIP 52.62, the frequency of lipiarmycin-resistant bacteria was 100-fold higher than the frequency observed for bacteria with the wild-type fragment.

By analogy with the three-dimensional structure of the *Thermus aquaticus* enzyme (3), R326 is located in proximity to region 3.2 of σ, which in turn occupies the same space as the exiting RNA transcript (3). This could delineate a new binding site for transcription inhibitors.

TABLE 1. Antibiotic resistance and transcription activity of Lprm-resistant *Bacillus subtilis*

Strain	MIC (μg/ml)[a]						Transcription activity[b] (IC_{50} [μg/ml])		Change	
	Lprm	RIF	STL	ERY	NOV	FUS	RIF	Lprm	Mutation	Codon
mut1	>100	0.01	50	0.05	0.09	1.5	0.01	50	R326 to L	CTT
mut2	>100	0.01	50	0.05	0.09	1.5	0.02	35	R326 to L	CTT
mut3	>100	0.2	50	0.05	0.78	12.5	0.03	4	None	CGT
mut4	>100	0.1	50	0.05	0.78	25	0.01	3	None	CGT
mut5	>100	0.2	50	0.05	0.78	12.5	0.01	4	None	CGT
mut6	>100	0.2	50	0.05	0.78	12.5	0.02	2	None	CGT
mut7	>100	0.2	50	0.05	0.78	25	0.01	5	None	CGT
mut8	>100	0.1	50	0.05	0.78	12.5	0.02	4	None	CGT
mut9	>100	0.1	50	0.05	0.78	25	0.01	4	None	CGT
mut10	>100	0.4	50	0.05	0.78	12.5	0.01	2	None	CGT
CIP 52.62	6.25	0.02	50	0.05	0.09	1.5	0.01	3	None	CGT

[a] The MICs were determined in triplicate by a broth microdilution method in 96-well microtiter plates as described by the CLSI (formerly NCCLS) guidelines (4). The following antibiotics were used: Lprm, rifampin (RIF), streptolydigin (STL), erythromycin (ERY), novobiocin (NOV), and fusidic acid (FUS).

[b] The transcription activities of Lprm-sensitive and -resistant mutants were measured by the method of Sonenshein et al. (6) on toluene-permeabilized *B. subtilis* cells. The template for these reactions was the endogenous *B. subtilis* DNA, and the concentration necessary to inhibit 50% of the [5-^{3}H]UTP incorporation was calculated using the LSW data analysis tool (MDL, San Leandro, CA).

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