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COMMUNICATIONS TO THE EDITOR

Resistance to Paromomycin is Conferred by *rpsL* Mutations, Accompanied by an Enhanced Antibiotic Production in *Streptomyces coelicolor* A3(2)

Sir:

Aminoglycoside antibiotics interfere with bacterial protein synthesis by binding to the 30S ribosomal subunit[1,2]. Their binding is known to stabilize the tRNA-mRNA interaction in the A-site by decreasing the tRNA dissociation rates[3]. Eventually, aminoglycoside antibiotics cause a decrease in translational accuracy and inhibit translocation of the ribosome. Such detrimental effects of aminoglycoside antibiotics can be circumvented by mutations altering 16S rRNA[4~6] or certain ribosomal proteins[6~9]; these mutations have been found in either the nucleotide 530 or 915 regions of 16S rRNA or in ribosomal protein S12.

We previously reported that certain mutations in *rpsL* (encoding the ribosomal S12 protein) not only confer resistance to streptomycin but also activate the production of secondary metabolites (antibiotic production) in various bacteria[10,11]. We were interested in determining whether aminoglycoside antibiotics other than streptomycin exert similar stimulatory effects on secondary metabolism. In the present paper, we report that a mutation in *rpsL* conferring resistance to paromomycin in *Streptomyces coelicolor* is accompanied by antibiotic overproduction.

Streptomyces coelicolor A3(2) strain 1147, a prototrophic wild type strain, was used in this study. It produces a blue pigmented antibiotic, actinorhodin[12]. The compositions of GYM medium[13] and R4 medium[10] were described previously. Spontaneous paromomycin-resistant mutants were obtained as colonies that grew within 7 days at 30°C after spores were spread on GYM agar containing various concentrations of paromomycin. Actinorhodin production was determined by measuring the optical density of culture filtrates at 600 nm. To characterize the mutations, the *rpsL* genes of parental or mutant strains were amplified from their genomic DNAs by PCR, as described previously[10] and the nucleotide sequences of the PCR products were determined (Model ABI310, PE Biosystems).

Mutants developed on plates containing 1 or 2 μg/ml of paromomycin at a frequency of 10^{-7} or 10^{-8}, respectively. Out of 77 randomly selected paromomycin resistant mutants, 4 strains, designated as KO-347~350, were found to produce actinorhodin in 7 to 10-fold greater amounts than the parent strain (Table 1). In our previous studies[10,11,14] we established that an antibiotic-overproducing characteristic frequently appears together with the mutation within the *rpsL* gene. To address this relationship we examined for the presence of mutations in *rpsL*. Strikingly, all antibiotic-overproducing mutants (Class I) examined had *rpsL* mutations, resulting in the alteration of amino acid residue 91 from proline to serine (Table 1). In contrast, another group of paromomycin resistant mutant, (Class II; KO-351~356) which do not overproduce antibiotic, was found to have no mutation within the *rpsL* gene. It should be pointed out that Class I mutants displayed a higher level of resistance to paromomycin than that of Class II mutants (Table 1). Thus, these findings indicate that the phenotypic characteristics of paromomycin resistant mutants accurately correlate with their genetic characteristics. Moreover, the Class I mutants revealed an apparent cross-resistance (20-fold) to streptomycin, while the Class II mutants showed only a slight (2 to 3-fold) resistance. Cross-resistance to other aminoglycoside antibiotics (hygromycin B, gentamicin, and kanamycin) was much less pronounced (Table 1). Surprisingly, Class I mutants with higher level of resistance to paromomycin revealed an increased (two-fold) sensitivity to neomycin.

Paromomycin belongs to the neomycin group of aminoglycosides which is structurally different from streptomycin. Aminoglycosides are composed of aminosugars attached to a deoxystreptamine ring. Rings I and II within the molecule are the most commonly found in the aminoglycosides. Recently, it was reported that functional groups such as OH or NH_2 in rings I and II are responsible for their characteristic miscoding pattern[15]. Streptomycin does not contain such functional groups within the molecule and thus its miscoding pattern is considered to be distinct from that of other aminoglycosides[6].

There are an increasing number of publications which report the interaction between aminoglycoside antibiotics and 16S rRNA (reviewed by MOAZED *et al.*[4]). The results of chemical footprinting have led to the suggestion that the

Table 1. Characterization of paromomycin resistant mutants of *S. coelicolor*.

Strain		Actinorhodin productivity[a]	Mutation in *rpsL* gene [b] (Amino acid position and exchange)	Level of resistance (μg ml^{-1}) to [c]					
				Par [d]	Str	Neo	Hyg	Gen	Kan
S. coelicolor 1147		+	None [e]	0.1	1	0.5	1	0.1	0.1
Mutant class I	KO-347	+++	C-271 -> T (Pro-91 -> Ser)	0.75	20	0.25	2	0.5	0.1
	KO-348	+++	C-271 -> T (Pro-91 -> Ser)	0.75	20	0.25	1.5	0.3	0.1
	KO-349	+++	C-271 -> T (Pro-91 -> Ser)	0.75	20	0.25	2	0.5	0.1
	KO-350	+++	C-271 -> T (Pro-91 -> Ser)	0.75	20	0.25	2	0.5	0.1
class II	KO-351	+	None	0.3	3	0.75	1	0.2	0.25
	KO-352	+	None	0.3	2	0.75	1	0.2	0.25
	KO-353	+	None	0.3	2	0.75	1	0.2	0.25
	KO-354	+	None	0.3	2	0.75	1	0.2	0.25
	KO-355	+	None	0.3	3	0.75	1	0.2	0.25
	KO-356	+	None	0.3	3	0.75	1	0.2	0.25

a) Determined after 8 days of culture at 30℃ in R4 medium. + and +++ indicate OD_{600}=0.1 to 0.24 and OD_{600}= 1.4 to 2.1, respectively.

b) Numbering originates from the start codon (GTG) of the open reading frame.

c) Determined after 3 days of incubation at 30℃ on GYM agar.

d) Par : paromomycin, Str : streptomycin, Neo : neomycin, Hyg : hygromycin, Gen : gentamicin, Kan : kanamycin

e) Mutations were not detected within the *rpsL* gene.

neomycin group of antibiotics interact with multiple sites in the conserved A-site region of *E. coli* 16S rRNA[4]. However, paromomycin, a member of the neomycin group of antibiotics, has been demonstrated to bind only at a single site (the A 1408–G 1494 region) which has been well characterized by structural studies[4,6,15,17]. The previously known mutations which confer paromomycin resistance are located at nt1409, and 1491 (numbered according to *E. coli* 16S rRNA), respectively[4,18,19]. In contrast, it has been proposed that streptomycin binds at multiple sites (nt915 and 1410 regions) in 16S rRNA, on the basis of results from chemical protection and cross-linking experiments[4,20]; the primary action site of streptomycin appears to be the nt915 region[4]. In agreement with these suggestions, all 16S rRNA mutations that confer streptomycin resistance that have been found so far were located in the nt915 and 530 regions[6]. The nt530 region that forms the characteristic loop within the 16S rRNA is one of the most highly conserved regions and is itself located alongside sites that are protected by aminoacyl-tRNA, bound in the A-site[6,21]. Paromomycin causes a conformational change in the nt530 region, and its conformational change leads to enhanced reactivity of C-535 with chemicals in footprinting experiments[4].

Ribosomal protein S12 has been shown to be adjacent to the 530 loop[22]; mutations in this protein (and S4) have been reported to influence the higher-order structure of 16S rRNA[23]. The three-dimensional structure of the ribosome is recently getting clear and the structural studies provide evidence that some ribosomal proteins are directly involved in ribosomal function[24]. Amino acid alterations in the positions Lys-43, Arg-86, Lys-88, and Pro-91 in S12 have been previously reported to have conferred streptomycin resistance in *E. coli*, *B. subtilis*, *Mycobacterium tuberculosis*, and *S. coelicolor*[6,10,11,14,25,26]. Our principal finding in this study was that resistance to paromomycin, an antibiotic which is distinct from streptomycin in both structure and function, is conferred by a mutation in the

ribosomal protein S12. This is the first report which demonstrates that resistance to paromomycin is conferred not only by a 16S rRNA mutation but also by a mutation in a ribosomal protein. It should be noted that *E. coli* strains dependent on paromomycin (and therefore resistant) were previously reported by GORINI *et al.*[27]; paromomycin also induces mistranslation *in vivo* and can phenotypically suppress nonsense mutations.

The position Pro-91, which had been found in this study, had already been established as the sited mutations conferring streptomycin resistance in *E. coli*[28] and in chloroplasts[7,29]; but it has not, so far, been found among the streptomycin-resistant mutants from *S. coelicolor*. It is conceivable that a mutant S12 protein altered at the Pro-91 position prevents the conformational change in the 530 loop, an event which normally occurs in wild type ribosomes in the presence of paromomycin. Our present finding may offer a feasible system for studying the details of the mechanism of action of the neomycin group of aminoglycosides.

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