



Environmental and genetic factors affecting mutability to aminoglycoside antibiotics among *Escherichia coli* K12 strains

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Abstract

Environmental and genetic factors affecting the in vitro spontaneous mutation frequencies to aminoglycoside resistance in *Escherichia coli* K12 were investigated. Spontaneous mutation frequencies to kanamycin resistance were at least 100 fold higher on modified Luria agar (L2) plates, when compared to results obtained in experiments carried out with Nutrient agar (NA) plates. In contrast to rifampicin, the increased mutability to kanamycin resistance could not be attributed to a mutator phenotype expressed by DNA repair defective strains. Kanamycin mutant selection windows and mutant preventive concentrations on L2 plates were at least fourfold higher than on NA plates, further demonstrating the role of growth medium composition on the mutability to aminoglycosides. Mutability to kanamycin resistance was increased following addition of sorbitol, suggesting that osmolarity is involved on the spontaneous mutability of *E. coli* K12 strains to aminoglycosides. The spontaneous mutation rates to kanamycin resistance on both L2 and NA plates were strictly associated with the selective antibiotic concentrations. Moreover, mutants selected at different antibiotic concentrations expressed heterogeneous resistance levels to kanamycin and most of them expressing multiple resistance to all tested aminoglycoside antibiotics (gentamicin, neomycin, amikacin and tobramycin). These results will contribute to a better understanding of the complex nature of aminoglycoside resistance and the emergence of spontaneous resistant mutants among *E. coli* K12 strains.

Key words: antibiotic resistance, aminoglycosides, *Escherichia coli* K12, mutator strains, spontaneous mutation frequencies.

Received: October 21, 2002; accepted: March 10, 2003.

Introduction

Understanding the nature of antibiotic resistance among different bacterial species and the proposal of new strategies to reduce the emergence of resistant strains represent great challenges to modern microbiologists (Neu, 1992; Davies, 1994). Antibiotic resistance arises among bacterial populations by endogenous or exogenous mechanisms. Exogenous resistance requires the horizontal acquisition of genes carried by mobile genetic elements, such as plasmid and transposons, or by recombination of foreign DNA into the chromosome; whereas endogenous antibiotic resistance involves the emergence of spontaneous mutations, which may vary according to both environmental factors and intrinsic bacterial genetic features (LeClerc *et al.*, 1996; Martinez and Baquero, 2000; Drlica, 2001).

The spontaneous rates of antibiotic resistance among bacterial populations reflect the interplay of intrinsic genetic features of the strain and exogenous environmental

factors capable of changing the physiological condition of the cell. Malfunction of genes controlling DNA repair mechanisms, such as those affecting the *mutS* gene, which participate in methyl-directed mismatch DNA repair and the *mutT* and *mutY* genes, encoding enzymes involved in oxidized guanine repair, can drastically affect the spontaneous mutation frequencies among different bacterial species (Bridges, 1995; LeClerc *et al.*, 1996; Matic *et al.*, 1997). Moreover, composition of the growth medium, selective antibiotic concentrations, environmental stresses and the mutagenic nature of some antibiotics represent additional environmental factors that can affect the in vitro spontaneous mutability to antibiotic resistance (Foster, 1993; Ren *et al.*, 1999; Martinez and Baquero, 2000). A better knowledge of the in vitro conditions affecting the spontaneous mutation frequencies to antibiotic resistance represents, therefore, an important step for the understanding of antibiotic resistance among different bacterial species.

Aminoglycosides are bactericidal broad-spectrum antibiotics able to impair protein synthesis through binding to ribosomes and disruption of cell envelope permeability (Busse *et al.* 1992; Mingeot-Leclercq *et al.*, 1999). In spite

of the broad activity toward most gram-negative pathogens, the emergence of resistant strains has significantly reduced their clinical relevance (Davies and Wright, 1997). Although the expression of drug modifying enzymes represents the most clinically relevant mechanism, epidemiological surveys have detected aminoglycoside resistant strains with reduced cell envelope permeability among different bacterial species (Price *et al.*, 1981; Aminoglycoside Resistance Study Group, 1994). Unlike strains expressing drug modifying enzymes, such strains are resistant to all aminoglycosides and probably arise as a consequence of spontaneous mutations at chromosomal loci present either in laboratory or wild bacterial strains (Miller *et al.*, 1995; Acosta *et al.*, 2000).

In this work we investigated some environmental and genetic factors involved in the incidence of spontaneous mutations leading to aminoglycoside resistance among *E. coli* K12 strains. The results demonstrate the complex nature of the spontaneous mutability to aminoglycoside antibiotics and suggest how it can affect the emergence of multiple resistance among bacterial populations.

Materials and Methods

Bacterial strains

Seven *E. coli* K12 strains were used in the present study. Four strains were proficient in DNA repair (CSH109, CC104, BB, and J53), one strain (CSH115 *mutS*) was deficient in methyl-directed mismatch DNA repair, one strain (CHS114 *mutT*), was deficient in the 8-oxoguanine error avoidance system and another (JM109) was mutated at the *recA* gene, which controls the SOS repair. The bacterial strains and their major genetic characteristics are listed in Table 1.

Growth conditions

Strains were grown overnight in aerated conditions at 37 °C. Bacterial cultures were prepared either in double strength Luria broth (2% tryptone, 1% yeast extract, 1% NaCl) or nutrient broth (3% w/v). Double strength L agar (L2) or nutrient agar (NA) plates were prepared following addition of agar at a final concentration of 1.5%. All growth

medium components were purchased from Difco. Kanamycin was used at various final concentrations and rifampicin was used at a final concentration of 60 µg/mL. Other aminoglycosides were tested at various concentrations: 10 µg/mL for tobramycin and gentamicin, 20 µg/mL for neomycin and 5 µg/mL for amikacin. All antibiotics were purchased from Sigma.

Spontaneous mutation frequencies

One bacterial colony was suspended in 10 mL of Luria or Nutrient broths and grown at 37 °C overnight under agitation. Cultures were serially diluted in phosphate buffered saline (PBS, all components were purchased from Merck) and 100 µL aliquots were spread on L2 or NA plates and then incubated at 37 °C for 48 h. Aliquots of undiluted cultures as well as those diluted to 10⁻¹ and 10⁻² were plated on antibiotic containing plates for titer determination of the kanamycin resistant colonies. The numbers of viable cells of each culture were determined after plating 100 µL aliquots of PBS-diluted samples on plates without added antibiotic. Mutation frequencies were expressed as the number of antibiotic resistant colonies per 10⁸ viable cells. Experiments were repeated independently at least three times using two plates for each antibiotic concentration.

Minimal inhibitory concentrations (MIC) and mutant preventive concentrations (MPC)

MICs for the various antibiotics were determined with L2 or NA plates containing serial two-fold dilutions of the tested antibiotic. Aliquots of PBS-diluted cultures containing 10⁴ to 10⁵ CFU of each tested strain were spread on plates and incubated at 37 °C for 24 h. MPCs, defined as the lowest inhibitory antibiotic concentration allowing detection of resistant colonies (Sindelar *et al.*, 2000), were determined after spreading 100 µL aliquots containing 10⁹ cells on plates with two-fold serial dilutions of kanamycin and incubation at 37 °C for 36 h.

Results

Different *E. coli* K12 strains, not necessarily isogenic, proficient (CSH109, CC104, J53, and BB) or deficient (CSH114, CSH115, and JM109) in DNA repair displayed spontaneous mutation frequencies to kanamycin resistance, in NA plates containing 60 µg/mL, at a range below the detection limit of our assays (2 × 10⁻⁹). When the same experiments were performed with LA plates containing the same selective antibiotic concentration (except the J53 strain which was spread on plates containing 20 µg/mL of kanamycin, due to the inherent lower sensitivity of the strain) the spontaneous mutation frequencies rose to values ranging from 2 × 10⁻⁷ to 7 × 10⁻⁷, values at least 100 fold higher than those attained in NA plates (Table 2). Two mutator strains, with inactive *mutT* (CSH14) or *mutS*

Table 1 - Bacterial strains used in the present study.

Strains	Major genetic features	Reference
CSH109	<i>ara</i> Δ(<i>gtp-lac</i>) 5 <i>rpsL</i>	Miller, 1992
CSH114	<i>mutT</i> derivative of CSH109	Miller, 1992
CSH115	<i>mutS</i> derivative of CSH109	Miller, 1992
CC104	<i>ara</i> Δ(<i>gtp-lac</i>)5	Cupples and Miller, 1989
J53	<i>proA met</i>	Clowes and Hayes, 1968
BB	r ₃ ⁻ r _m ⁺ Su ⁺	Bullock <i>et al.</i> , 1987
JM109	<i>recA supE44 hscR17 thi-1</i> <i>gyrA relA1 thi ? (lac proAB)</i>	Yanish-Peron <i>et al.</i> , 1985

Table 2 - Spontaneous mutation frequencies \pm SD to antibiotic resistance of different *E. coli* K12 strains.

Strains	Km ^R mutants per 10 ⁸ cells ¹		Rif ^R mutants per 10 ⁸ cells ²	
	L2	NA	L2	NA
DNA repair proficient strains				
CSH109	21 \pm 2.6	ND ³	1.2 \pm 0.3	ND
CC104	40 \pm 5.2	ND	0.4 \pm 0.8	ND
J53	18 \pm 3.2	ND	1.1 \pm 0.2	ND
BB	52 \pm 11.3	ND	3.3 \pm 0.5	2.6 \pm 1.1
DNA repair deficient strains				
CSH114 (<i>mutT</i>) [*]	54 \pm 7.8	ND	150 \pm 28	20 \pm 3.5
CSH115 (<i>mutS</i>) [*]	67 \pm 4.5	ND	154 \pm 34	90 \pm 10.5
JM109 (<i>recA</i>)	36 \pm 4.2	ND	6.1 \pm 1.4	0.7 \pm 0.2

1 - frequencies of kanamycin-resistant colonies determined on plates containing 60 μ g/mL kanamycin with the exception of the J53 strain plated in media containing 20 μ g/mL kanamycin. Frequency of Km^r mutants expressed as average (\pm SD) number of Km^r resistant colonies per 10⁸ viable cells.

2 - frequencies of rifampicin-resistant colonies determined in plates containing 60 μ g/mL of rifampicin. Frequencies of Rif^r mutants expressed as average (\pm SD) number of Rif^r colonies per 10⁸ viable cells.

3 - ND - frequency of spontaneous resistant mutants below 2×10^{-9} .

* - a mutator phenotype was assigned to strains expressing spontaneous mutation frequencies which were 10 to 100 fold higher than those expressed by the parental or DNA repair proficient strains.

(CSH15) genes, showed spontaneous mutation frequencies to kanamycin resistance on L2 plates, up to 3 times higher than that the parental strain (CSH109), which were similar to frequencies expressed by other DNA repair proficient strains, as CC104 and BB. No differences were noted between the kanamycin resistance spontaneous mutation frequencies of the *recA* (JM109) strain, deficient in the SOS repair pathway, and DNA repair proficient strains (Table 2). On the other hand, experiments performed with an unrelated antibiotic, rifampicin, showed that the frequencies of spontaneous resistant mutants in L2 plates ranged from 10⁻⁸ to 4 \times 10⁻⁹, which were up to tenfold higher than the values found on NA plates for the DNA repair proficient strains. The CSH14 and CSH15 strains clearly expressed their mutator phenotypes when tested for mutability to rifampicin, which were at least 2 orders of magnitude higher than those detected with the parental strain and other DNA proficient strains, on both L2 and NA plates (Table 2). Such results show that spontaneous mutability to kanamycin resistance did not result from a mutator phenotype expressed by the tested strains, but it is apparently dependent on the composition of the assay medium irrespective of the genetic background of the tested bacterial strains.

Composition of the growth medium affected both MIC and MPC for kanamycin of the tested *E. coli* K12 strains. MIC values for kanamycin on L2 plates for the

DNA repair proficient strains (CSH109, CC104, J53 and BB) ranged from 16 to 8 μ g/mL, which were four times higher than the values determined in NA plates ranging from 4 to 2 μ g/mL (Table 3). Similarly, MPC values for kanamycin varied from 128 to 64 μ g/mL in assays carried out on L2 plates, while on NA plates the values were reduced to 8 or 4 μ g/mL. The MPC/MIC ratios, which expressed the mutant selection windows as multiples of the MIC values, showed that the kanamycin concentration range allowing resistant mutant selection on L2 plates was four times higher than that found on NA plates for all tested *E. coli* strains (Table 3).

The selective antibiotic concentration is an environmental factor known to affect the spontaneous mutability to antibiotic resistance among different bacterial species (Martinez and Baquero, 2000). Experiments carried out with CSH109 and J53 strains showed that the spontaneous frequencies to kanamycin resistance are indeed strictly dependent on the selective antibiotic concentration irrespective of the composition of the growth media. As shown in Table 4, frequencies of kanamycin resistant mutants may increase up to 1,000 fold according to the selective antibiotic concentration either on L2 or NA plates (Table 4). Moreover, addition of 0.5 M sorbitol to NA plates enhanced the spontaneous mutation frequency to kanamycin resistance of the J53 strain to values which were up to 30 fold higher than those determined at the same selective antibiotic concentration on NA plates (Table 4). These results indicate that *in vitro* mutabilities to kanamycin resistance among *E. coli* K12 strains are dependent on both selective concentration and osmolarity of the growth medium.

The enhanced spontaneous mutation rates observed at lower kanamycin concentrations suggest that different cellular targets, conferring distinct antibiotic resistance levels, were affected during the *in vitro* selection. In order to evaluate the heterogeneous nature of spontaneous kanamycin resistance mutants, the J53 strain was submitted to selections carried out with L2 plates containing different

Table 3 - MIC and MPC values for kanamycin expressed by DNA repair proficient *E. coli* K12 strains in L2 and NA plates.

Strains	MIC (μ g/mL) ¹		MPC (μ g/mL) ²		MPC/MIC ratio ³	
	L2	NA	L2	NA	L2	NA
CSH109	16	4	128	8	8	2
CC104	16	4	128	8	8	2
J53	8	2	64	4	8	2
BB	16	4	128	4	8	2

1 - MIC determined as the kanamycin concentration resulting in complete growth inhibition of the strains on L2 or NA plates;

2 - MPC determined as the kanamycin concentration resulting in mutation frequencies below 0.2 colonies per 10⁸ viable cells;

3 - MPC/MIC ratios representing the concentration ranges, measured as multiples of MIC values, in which spontaneous kanamycin resistant colonies could be isolated.

kanamycin concentrations (10, 20 and 40 $\mu\text{g}/\text{mL}$). As shown in Figure 1, the MIC values of the kanamycin resistant mutants selected at different selective concentrations were quite heterogeneous with values ranging from 50 $\mu\text{g}/\text{mL}$ to over 200 $\mu\text{g}/\text{mL}$. There was also a clear correlation between the selective antibiotic concentration and the MIC values expressed by the selected mutants. For example, only 12% of the mutants selected on L2 plates containing 10 $\mu\text{g}/\text{mL}$ kanamycin expressed MIC values above 200 $\mu\text{g}/\text{mL}$, while 42% of those selected at 40 $\mu\text{g}/\text{mL}$ of kanamycin were resistant to antibiotic levels over 200 $\mu\text{g}/\text{mL}$ (Figure 1). Moreover, most of the spontaneous mutants selected at 10, 20 or 40 $\mu\text{g}/\text{mL}$ kanamycin expressed multiple resistances to aminoglycosides, as tested for gentamicin, amikacyn, neomycin and tobramycin. Screening of spontaneous J53 mutants selected on L2 plates containing kanamycin at final concentrations of 10, 20 and 40 $\mu\text{g}/\text{mL}$ expressed multiple resistances to aminoglycosides at frequencies of 96%, 92%, and 88%, respectively (Figure 2). Taken together these results indicate

Table 4 - Spontaneous mutation frequencies \pm SD to kanamycin resistance of strains CSH109 and J53 at different selective antibiotic concentrations.

Strain	Medium	Selective concentration ($\mu\text{g}/\text{mL}$)	Km^{R} mutants per 10^8 cells ¹
CSH109	L2	40	160 \pm 31
		50	50 \pm 14
		60	12 \pm 19
		100	4.6 \pm 1.2
	NA	4	120 \pm 21
		5	72 \pm 17
		7	2 \pm 0.1
		8	ND ²
J53	L2	20	18 \pm 3.2
		30	7.5 \pm 1.2
		40	2 \pm 0.3
		50	ND
		NA	2
	NA	2.5	19 \pm 2.5
		3	3 \pm 0.6
		5	ND
	NA +	2	420 \pm 64
	0,5 M sorbitol	2.5	120 \pm 18
		3	81 \pm 13
5		2.1 \pm 0.1	

1 - frequencies of kanamycin resistant colonies calculated as: titer kanamycin resistant mutants/titer of the same culture in medium without added antibiotic;

2 - ND - frequency of spontaneous resistant mutants below 2×10^{-9} .

the heterogeneous nature of the spontaneously selected kanamycin resistant mutants among *E. coli* K12 strains and suggest that most of them were altered in cell envelope per-

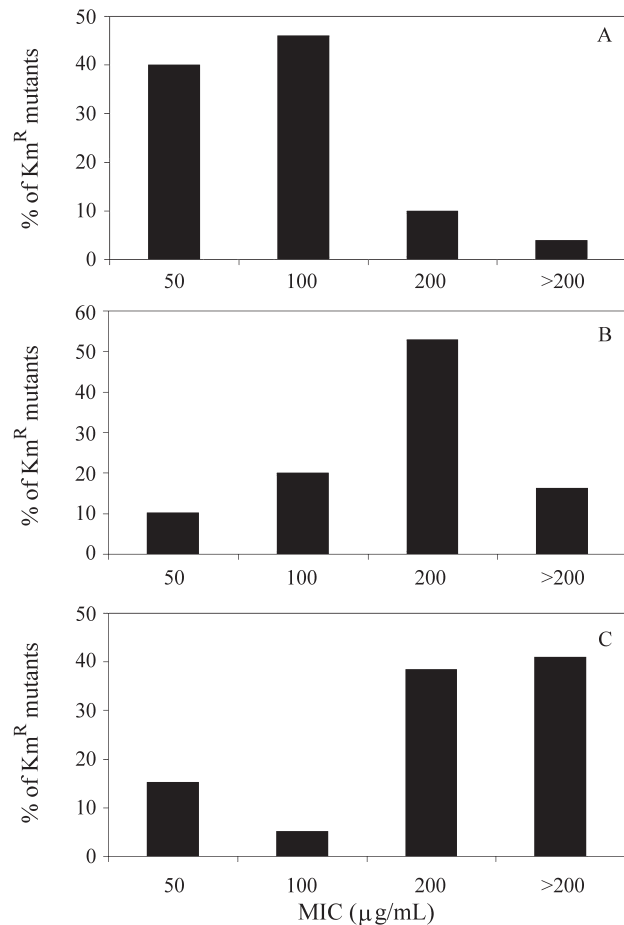


Figure 1 - MIC values of spontaneously selected kanamycin resistant mutants of *E. coli* K12 strain J53 selected at different antibiotic concentrations. Resistant mutants were selected on L2 plates containing 10 $\mu\text{g}/\text{mL}$ (A); 20 $\mu\text{g}/\text{mL}$ (B); and 40 $\mu\text{g}/\text{mL}$ (C) kanamycin. Values were expressed as percentage of mutants analyzed. Number of mutants analyzed in each group: 50 in A, 49 in B and 39 in C.

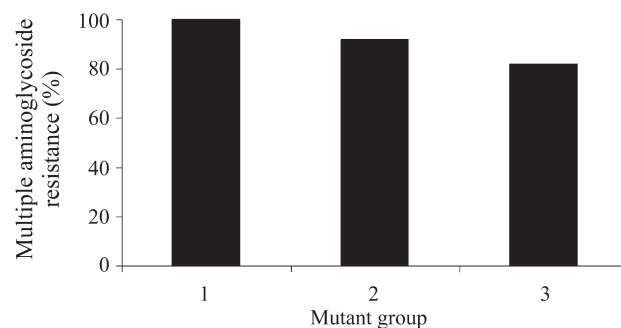


Figure 2 - Frequency of mutants, derived from the J53 strain, expressing multiple resistance to aminoglycosides (amikacin, kanamycin, gentamicin, neomycin, and tobramycin). Resistant mutants selected at different kanamycin concentrations were obtained as described in Figure 1 legend. Number of mutants analyzed in each group: 50 in A, 49 in B and 39 in C.

meability leading to the multiple resistance to aminoglycosides.

Discussion

Factors affecting the mutability of chromosomal genes conferring resistance to antibiotics have a potential role to contribute to the problem of multiple drug resistance among different bacterial species (Hakenbeck *et al.*, 1999; Hooper, 1999; Drlica, 2001). Moreover, the dynamic nature of spontaneous mutation rates, which are largely affected by environmental and genetic factors, claims for a better understanding of bacterial mutability for clinically relevant antibiotics (Martinez and Baquero, 2000).

In the present study, the spontaneous mutation frequencies to kanamycin resistance, among *E. coli* K12 strains, were shown to change according to the composition of the growth medium and the selective antibiotic concentration. So far, the factors involved on the spontaneous mutability to aminoglycoside antibiotics have been greatly overlooked, but previous evidences have demonstrated the strong influence of growth media composition on susceptibility to aminoglycoside (Gilbert *et al.*, 1971; Medeiros *et al.*, 1971; Rodriguez *et al.*, 1990). The present data deserve, therefore, particular attention since demonstration that in vitro spontaneous mutation frequencies of *E. coli* K12 strains to aminoglycoside antibiotics can be enhanced according to the composition of the growth medium and selective antibiotic concentration, suggests that, under specific in vivo conditions, where lower antibiotic concentrations and high osmotic conditions might prevail, selection of chromosomal mutants conferring multiple resistance to aminoglycosides may contribute to the problem of multiple antibiotic resistance among clinically relevant bacterial species.

The composition of the growth medium is known to affect the sensitivity of several gram-negative bacterial species to aminoglycosides (Gilbert *et al.*, 1971; Medeiros *et al.*, 1971; Rodriguez *et al.*, 1990). Growth medium ionic strength, as well as the concentration of divalent cations, has long been known to affect in vitro susceptibility of gram-negative bacteria to aminoglycoside, such as *Pseudomonas aeruginosa* to gentamicin (Medeiros *et al.* 1971). A previous report had also emphasized the effect of growth medium osmolarity on the susceptibility of several gram-negative bacterial to different aminoglycosides (Rodriguez *et al.*, 1990). Our results indicated that both sensitivity and spontaneous mutability to kanamycin resistance were altered according to the composition of the growth medium. The partial recovery of mutability to kanamycin resistance in NA medium following addition of 0.5 M sorbitol suggested that growth medium osmolarity is indeed an environmental factor capable of modulating the spontaneous mutation frequency to aminoglycoside antibiotics.

The correlation between susceptibility to aminoglycosides and spontaneous mutation frequencies to antibiotic resistance indicates that mutations conferring different antibiotic resistance levels may be selected at different selective concentrations. We can suggest, therefore, that growth conditions capable of increasing the resistance levels to aminoglycosides will also enhance the frequency of spontaneous resistant mutants, which can express cross-resistance to all aminoglycosides at clinically relevant concentrations. Further support for such hypothesis should await experiments carried out with wild type bacterial strains under various conditions known to affect the constitutive resistance to aminoglycosides.

The endogenous resistance to aminoglycosides is a complex process, which involves several cellular components located either in the ribosome machinery or in the cell envelope (Bryan and Kwan, 1983; Taber *et al.*, 1987; Davies and Wright, 1997; Kotra *et al.*, 2000). Spontaneous mutations at several genes, such as those encoding ribosomal proteins, ribosomal RNA species, respiratory chain components, H⁺-ATPase subunits, oligopeptide permease and enzymes involved in lipopolisaccharide biosynthesis, are all known to confer resistance to aminoglycosides (Taber and Halfenger, 1976; Hancock, 1981; Hancock *et al.*, 1991; Muir *et al.*, 1985; Muir and Wallace, 1982; Plate *et al.* 1986; Kashiwagi *et al.*, 1992, 1998). Thus, the high mutation rates detected at low selective kanamycin concentrations most probably reflect the multiplicity of targets, which can confer a resistance phenotype once altered by a mutational event. Of particular relevance are the genes known to confer multiple resistance to aminoglycosides, such as those encoding proteins involved on the generation of the membrane electric potential and those affecting the expression and functioning of the major oligopeptide uptake system (Taber *et al.*, 1987; Kashiwagi *et al.*, 1992, 1998). In contrast to strains expressing aminoglycoside-modifying enzymes, such mutants can acquire resistance to all available aminoglycosides in a single mutational event and may be detected at high frequencies either in laboratory or wild type *E. coli* strains (Acosta *et al.*, 2000).

Mutator genotypes increase mutation rates by 10-100 fold among bacterial populations of different bacterial species both in nature and in the laboratory (LeClerc *et al.*, 1996; Matic *et al.*, 1997). In our experiments we could not attribute any major role of mutator genotypes to the enhanced spontaneous mutation frequencies to kanamycin resistance in selections carried out at L2 media. The multiple target nature of the aminoglycoside resistance may explain the reduced effect of the mutator phenotype expressed by *mutS* or *mutT* *E. coli* strains on the mutability to kanamycin resistance. However, we cannot disregard the fact that the nature of the tested antibiotics could affect the in vitro mutability expressed by the two mutator strains.

The problem of multiple resistance to aminoglycoside antibiotics among clinically relevant bacteria has

been a major concern among physicians and microbiologist during the last several decades (Davies, 1994; Davies and Wright, 1997). Although much attention has been given to the identification and characterization of strains able to encode aminoglycoside-modifying enzymes, epidemiological surveys carried out in different countries around the world have emphasized the clinical relevance of strains expressing multiple resistance to aminoglycosides due to impermeability of the cell envelope (Price *et al.*, 1981; Aminoglycoside Resistance Study Group, 1994). Our results, although based on *E. coli* K12 strains, represent the first experimental evidence that spontaneous mutations conferring multiple resistances to aminoglycosides can arise at high frequencies under conditions of low selective antibiotic concentrations and specific growth conditions, as high osmolarity environments. Indeed, as previously demonstrated by our group (Acosta *et al.* 2000), most of these mutants are affected on the expression of the oligopeptide-binding protein A, the periplasmic component of the major peptide uptake system, which has also been shown to mediate uptake of aminoglycosides in *E. coli* K12 strains (Kashiwagi *et al.*, 1992, 1998). Such conditions can be found during *in vivo* growth of most human gram-negative pathogens, as in the intestinal environment. More detailed analyses of aminoglycoside mutability expressed by wild bacterial strains and a better knowledge of the mechanisms involved in aminoglycoside resistance may help to elucidate whether the spontaneous mutational events, observed in *E. coli* K12 strains, can contribute to the incidence of strains expressing multiple resistance to aminoglycosides.

Acknowledgments

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq). We gratefully acknowledge the helpful technical assistance of Maria Normandia de Lima e Kazui Higuchi.

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Editor: Carlos F.M. Menck