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Heterogeneous Macrolide Resistance and Gene Conversion in the Pneumococcus

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A macrolide-resistant clinical isolate of *Streptococcus pneumoniae* with 23S rRNA mutations showed a heterogeneous phenotype and genotype. The mutant 23S rRNA genes from this isolate transformed susceptible strain R6 to resistance. Culture of resistant strain R6 in the absence of antibiotic pressure showed gene conversion to occur between the four 23S rRNA alleles, resulting in reversion to susceptibility with the resistant phenotype showing a fitness cost. These data explain the disappearance on subculture of heterogeneous macrolide resistance in the pneumococcus.

Two predominant macrolide resistance mechanisms exist in the pneumococcus: target site modification by acquisition of *erm*(B) or, rarely, *erm*(A) (18), resulting in macrolide-lincosamide-streptogramin B resistance (22), and drug efflux due to *mef*(A), resulting in resistance to 14- and 15-membered macrolides (17). Strains that carry both mechanisms have been identified previously (9). Target site modification by mutations in the genes of 23S rRNA and ribosomal proteins L4 and L22 have more recently been found to cause macrolide resistance (2, 3, 19, 20, 23). The rRNA genes differ from most prokaryotic chromosomal genes in that they are present in multiple copies in the genome (5). In *Streptococcus pneumoniae* there are four copies of the *rrn* operon (5, 19). Multiple copies maintain extremely high homogeneity by gene conversion or nonreciprocal recombination (4, 6). Gene conversion is the homologous recombination between mutant and wild-type alleles of a gene. Where resistance mutations occur in genes present in multiple copies, such as the 23S rRNA genes in macrolide resistance, the study of resistance mechanisms is complicated by gene conversion. In this study the implications of gene conversion for macrolide resistance due to heterologous 23S rRNA mutations is investigated.

A clinical isolate of *S. pneumoniae* (PU1004017) initially identified as highly resistant to macrolides (drug MICs: erythromycin [ERY], 128 μg/ml; clarithromycin, >256 μg/ml; azithromycin, >256 μg/ml) and susceptible to clindamycin and telithromycin was obtained from the PROTEKT surveillance study. Pneumococci were routinely cultured at 37°C in 5% CO_2 on Mueller-Hinton agar (MHA) supplemented with 5% horse blood. MICs were determined by the agar dilution method according to CLSI (formerly NCCLS) guidelines (11) and the Etest (AB Biodisk, Solna, Sweden). Chromosomal DNA was extracted as previously described (14). PCR-based methods were used to screen for *erm*(B) and *mef*(A) (16). Genes encoding L4 and L22 and all four alleles of 23S rRNA were amplified according to previously described methods (3, 19). Amplified products were purified with the QIAquick gel extraction kit (QIAGEN Ltd., Surrey, United Kingdom). DNA sequencing was performed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems Model 310 automated DNA sequencer. ERY Etests showed a heterogeneous phenotype. A zone of inhibition was observed for isolate PU1004017 as is typical of a susceptible strain; however, satellite colonies occurred to a MIC of 256 μg/ml. PU1004017 tested negative for *erm*(B) and *mef*(A), and the genes encoding L4 and L22 were wild type. Sequencing of 23S rRNA genes revealed the mutation G2057A (*Escherichia coli* numbering) in all four alleles, while alleles 23S-1 and 23S-4 showed mixed bases at position 2059 (A/G). The heterogeneous phenotypic and genotypic data indicated that gene conversion may be taking place between the 23S rRNA alleles. Following subculture of the isolate in the presence of antibiotic (ERY, 64 μg/ml), the 23S rRNA genes were reanalyzed, and homogeneous results were obtained at both loci (G2057A [four out of four] and A2059G [three out of four]).

To confirm the role of these mutations in the macrolide resistance of the isolate, a nonencapsulated laboratory strain, *S. pneumoniae* R6, was made competent by culture in C medium (21) and transformation was performed as previously described (15). The 23S-1 allele of isolate PU1004017 (G2057A and A2059G) was used as donor DNA, and a transformant (T) was selected on MHA supplemented with 5% horse blood and containing ERY (16 μg/ml). Sequencing of the transformant 23S rRNA alleles revealed three fully mutated ($_{2057}$**AAG**$_{2059}$) and one wild-type ($_{2057}$**GAA**$_{2059}$) allele. However, as for isolate PU1004017, ERY Etests were inconsistent for the R6 transformant. Initial Etests showed a fully resistant phenotype; however, after 1 week of subculture on antibiotic-free media the transformant showed a heterogeneous phenotype with satellite colonies within the sensitive zone of inhibition, and after 10 days the transformant showed a sensitive phenotype.

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TABLE 1. Erythromycin MICs (in micrograms/milliliter) and 23S rRNA sequence data for R6 populations following continuous culture in the absence or presence of antibiotic[a]

Transformant population	Presence/absence of ERY	Day 0				MIC	Day 7				MIC	Day 14				MIC	Day 21				MIC
		23S rRNA gene no.					23S rRNA gene no.					23S rRNA gene no.					23S rRNA gene no.				
		1	2	3	4		1	2	3	4		1	2	3	4		1	2	3	4	
TA	−	M	M	M	M	>128	H	H	H	W	>128	W	W	W	W	0.03	W	W	W	W	0.03
	+						M	M	M	M	>128	M	M	M	M	128	M	M	M	M	>128
TB	−	M	M	M	M	>128	M	M	M	M	>128	M	M	M	M	>128	M	M	M	M	>128
	+						M	M	M	M	>128	M	M	M	M	>128	M	M	M	M	>128

[a] M, fully mutated at loci 2057 and 2059 ($_{2057}$AAG$_{2059}$); H, heterogeneous at loci 2057 and 2059 ($_{2057}$GAA$_{2059}$ and $_{2057}$AAG$_{2059}$); W, wild type at loci 2057 and 2059 ($_{2057}$GAA$_{2059}$).

The conversion of the 23S genes between the wild-type and mutated forms was further investigated. The transformant was initially subcultured three times over a 1-week period on ERY plates (64 μg/ml) to obtain a uniform culture, which showed all four 23S rRNA alleles mutated ($_{2057}$**AAG**$_{2059}$). At this point two single colonies (TA and TB) were selected for further analysis. TA and TB were serially passaged 10 times over a 3-week period on MHA supplemented with 5% horse blood with or without ERY (64 μg/ml). On days 0, 7, 14, and 21, MICs were determined by the agar dilution method and the 23S rRNA genes were sequenced (Table 1). Subculturing of both TA and TB in the presence of antibiotic maintained all four alleles in the mutated form over the 3-week period, and both cultures remained macrolide resistant (ERY MIC, >128 μg/ml). However, after 1 week of subculturing TA in the absence of antibiotic, one allele had completely reverted to the wild type and the three remaining alleles showed heterogeneous sequences at locus 2057 (G/A) and locus 2059 (A/G). The drug still maintained its MIC of >128 μg/ml for the strain. After 2 weeks in the absence of antibiotic, all alleles had reverted to wild type and the MIC decreased to 0.03 μg/ml. The 23S alleles and MICs remained unchanged after the third week in the absence of the antibiotic. For TB, 3 weeks of subculture in the absence of ERY did not result in any change in the four mutated 23S rRNA alleles or the ERY MIC (>128 μg/ml).

Bacterial gene conversion has been shown to be involved in the development of antibiotic resistance (7). Under antibiotic pressure, wild-type alleles convert to the mutated forms by homologous recombination with mutated alleles (1, 8). This process is mediated by RecA (13). Pillai et al. (12) described a linezolid-resistant *Staphylococcus aureus* strain with a G2576U mutation in all five copies of 23S rRNA genes that displayed a stable resistant phenotype. However, Meka et al. (10) documented a linezolid-resistant *S. aureus* strain with one wild-type and four mutant alleles that reverted to susceptibility in the absence of antibiotic pressure. In our study, gene conversion complicated the study of macrolide resistance in isolate PU1004017. Both the original isolate and the resulting transformant showed a heterogeneous phenotype and genotype. Culture of the transformant containing three of four mutated alleles in the presence of ERY for 1 week resulted in conversion of all four alleles to the mutated form. Continuous culture in the presence of antibiotic maintained the mutations and macrolide resistance. However, when cultured in the absence of antibiotic, gene conversion started within 1 week (three passages), and complete reversion to susceptibility was observed within 2 weeks (six passages). This occurred in a far shorter period than that reported for an *S. aureus* isolate that reverted to susceptibility (maintaining one mutated copy) after 60 passages in antibiotic-free media (10). Intragenomic allelic exchange therefore appears to occur far more rapidly in *S. pneumoniae*. Although all four alleles of the transformant were mutated following culture in the presence of ERY, subculture of TA in the absence of antibiotic resulted in complete reversion of all four alleles to wild type, apparently without a wild-type allele present for homologous recombination. In contrast, TB, after 3 weeks of culture in antibiotic-free media, maintained the resistant phenotype. Complete conversion has been previously shown to result in stable resistance (1, 12), as was observed for TB. We therefore propose that TA may not have reached complete conversion, and wild-type copies of 23S rRNA genes may have been present in a small proportion of cells in comparison to the mutant alleles, such that they were not detected by sequencing.

Growth studies were performed on TA at 21 days of subculture either in the absence (TA−) or presence (TA+) of ERY. Glycerol stocks were inoculated into tryptone soy broth (1:50 dilution), and turbidity was monitored at 600 nm every 30 min for 12 h (Fig. 1). Sequencing of the 23S rRNA alleles at 0 h and 12 h confirmed that gene conversion did not occur for the

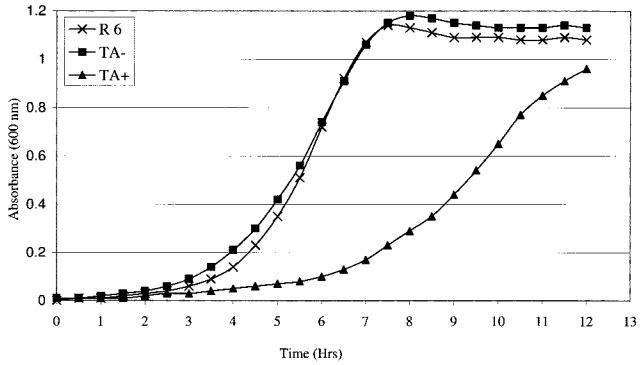

FIG. 1. Growth curves at 37°C for R6 compared to those of R6 transformants that had been subcultured for 21 days in the absence (TA−; $_{2057}$GAA$_{2059}$ [four out of four]) or presence (TA+; $_{2057}$AAG$_{2059}$ [four out of four]) of ERY (64 μg/ml).

duration of the growth curve. Mass doubling times (in minutes) during the exponential phase of duplicate growth curves were as follows: R6, 68.3; TA−, 84.9; TA+, 114.1. The reduced growth rate of TA+ (all four mutated genes) in comparison with TA− (wild type) suggests that the mutations are associated with a fitness cost. We therefore hypothesize that in the absence of antibiotic pressure, and provided that the strain has not reached complete conversion, pneumococci will revert to susceptibility in order to regain fitness by means of gene conversion.

Gene conversion in the absence or presence of antibiotics has implications for susceptibility testing as well as for the study of resistance mechanisms. We have shown in this study that routine subculture on antibiotic-free media can result in reversion to susceptibility. A resistant strain may therefore be inconsistently and incorrectly classified, with resulting implications for patient treatment. We have shown the ability of pneumococci to rapidly alter their resistance phenotypes and genotypes by means of gene conversion.

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