Single and dual mutations at positions 2058, 2503 and 2504 of 23S rRNA and their relationship to resistance to antibiotics that target the large ribosomal subunit

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Received 16 February 2011; returned 21 March 2011; revised 26 April 2011; accepted 1 June 2011

Objectives: To study mutations at positions A2058, A2503 and U2504 (Escherichia coli numbering) of 23S rRNA and their relationship to resistance to antibiotics that target the large ribosomal subunit.

Methods: Single and dual mutations at positions 2058, 2503 and 2504 of 23S rRNA were introduced into a Mycobacterium smegmatis strain with a single functional rRNA operon. MICs of macrolide, pleuromutilin, phenicol, lincosamide and oxazolidinone antibiotics were determined for the engineered mutants. The doubling times of the mutant strains were measured to investigate how the introduced mutations affected growth rate.

Results: Single mutations A2058G, A2503U and U2504G and double mutations A2058G–A2503U and A2058G–U2504G were successfully introduced. The A2058G mutation resulted in various levels of resistance to macrolides and clindamycin. The A2503U and U2504G mutations conferred resistance to valnemulin, chloramphenicol, florfenicol and linezolid. In addition, the A2503U mutant showed reduced susceptibility to the 16-membered macrolides tylosin, spiramycin and josamycin, and the U2504G mutant exhibited decreased susceptibility to spiramycin and josamycin. Moreover, the dual mutations A2058G–A2503U and A2058G–U2504G had co-effects on resistance to 16-membered macrolides.

Conclusions: 23S rRNA mutations A2058G, A2503U and U2504G play key roles in resistance to clinically useful antibiotics that target the large ribosomal subunit. Furthermore, the double mutations A2058G–A2503U and A2058G–U2504G have combined effects on resistance to 16-membered macrolides.

Keywords: 23S rRNA mutations, macrolides, peptidyl transferase centre, Mycobacterium smegmatis

Introduction

23S rRNA nucleotide A2058 (Escherichia coli numbering is used throughout this text) is a key determinant for the binding of macrolide antibiotics. Mutations at position 2058 are most commonly associated with macrolide/lincosamide/streptogramin B (MLSb) resistance in clinical isolates and laboratory strains of many bacteria species. In addition, the Erm methyltransferase, which modifies A2058 in 23S rRNA, similarly confers combined resistance to MLSb antibiotics.

A2053 and U2504 of 23S rRNA play key roles in resistance to several antibiotics that target the ribosomal peptidyl transferase centre (PTC). The recently described Cfr methyltransferase, which modifies A2053 in 23S rRNA, confers resistance to a broad range of drugs, including phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A. Our recent study showed that the A2053U mutation in 23S rRNA is associated with resistance to tiamulin, valnemulin, chloramphenicol, florfenicol and lincomycin in Mycoplasma gallisepticum. Furthermore, introduction of a single A2053G mutation into Mycobacterium smegmatis has shown that it could confer resistance to chloramphenicol and linezolid. U2504 belongs to the binding pockets of pleuromutilin, phenicol, lincosamide and oxazolidinone antibiotics. Introduction of the U2504G mutation into M. smegmatis showed that this mutation could confer resistance to valnemulin, chloramphenicol and linezolid. A recent study revealed that a U2504A mutation is responsible for high-level linezolid resistance in a clinical Staphylococcus epidermidis isolate. Moreover, many 23S rRNA mutations (such as G2032A, G2447U, U2500A, A2572U and G2576U) confer resistance to ribosomal PTC antibiotics by affecting the conformation and/or flexibility of nucleotide U2504.
In this study, we introduced single and double 23S rRNA mutations at positions 2058, 2503 and 2504 into a M. smegmatis strain engineered to possess a single functional rRNA operon. The susceptibilities of various antibiotics that bind at the ribosomal PTC and its associated region were determined to assess the importance of these key nucleotides in domain V of 23S rRNA for drug binding.

Materials and methods

Strains and plasmids

The strains and plasmids used in this study are summarized in Table 1. M. smegmatis strains were cultivated in Luria–Bertani (LB) medium supplemented with 0.05% Tween 80 (LB–Tween) to reduce cell clumping. E. coli strain XL1-Blue was used as a plasmid host and was grown in LB medium. For plasmid selection and maintenance, LB agar plates were supplemented with either 100 mg/L ampicillin or 100 mg/L hygromycin B.

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
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Str, streptomycin; Amp, ampicillin; Kan, kanamycin; Hyg, hygromycin B.

Construction of plasmids and transformation into M. smegmatis

An ~2.8 kb fragment of the functional rRNA (rrnA) was amplified from genomic DNAs of wild-type M. smegmatis using primers MSrrnAF (complementary to the sequences present in the functional operon but deleted from the inactivated operon) and MSrrnAR (Table S1, available as Supplementary data at JAC Online). The fragment was cloned into the pGEM-T Easy vector (Promega) to yield plasmid pMSrrnA.

Mutations were introduced into 1.3 kb fragments comprising the 3' end of 23S rRNAs by overlap extension PCR. Using the plasmid pMSrrnA as template, primers MSMFCX and MSMRXN (Table S1) were used in combination with mutagenic primers (Table S1) to introduce single mutations into the 1.3 kb fragments. The fragments were ligated into plasmid pMV361-Hyg⁸ cleaved with SpeI and either XhoI or CiaI. The double digestions deleted the aph cassette and the fragment ligations resulted in the following plasmids: pMV361Δkan-Hyg-rRNA2058G, pMV361Δkan-Hyg-rRNA2503U and pMV361Δkan-Hyg-rRNA2504G.

For the introduction of double mutations, plasmid pMV361Δkan-Hyg-rRNA2058G was used as a template and the procedure described above was used to introduce the additional A2503U and U2504G mutations. The resulting plasmids were pMV361Δkan-Hyg-rRNA2058G-2503U and pMV361Δkan-Hyg-rRNA2058G-2504G. Plasmid pMV361Δkan-Hyg-rRNA, which contained no mutations, was also constructed to serve as a control in the transformation experiments.

Transformation of plasmids into M. smegmatis mc²155 SMR5 rRNA⁻ was performed essentially as described previously,⁹ except that the strains were grown in LB–Tween medium. Primary selection was performed on agar plates containing hygromycin B at 100 mg/L. After 3–5 days of incubation, cells grown on LB plates containing hygromycin B were collected and applied to LB agar plates containing the following antibiotics: 128 mg/L erythromycin (for A2058G), 64 mg/L florfenicol (for A2503U and U2504G), and 128 mg/L erythromycin and 64 mg/L florfenicol (for A2058G–A2503U and A2058G–U2504G). Plates were incubated at 37°C for 3–5 days or until single colonies appeared. Single colonies were picked and purified for further investigation. The rDNA fragments of the functional rRNA operon (rrnA) were amplified with primers MSrrnAF and MSrrnAR and sequenced using internal primers MSABF and MSABR (Table S1) to verify that the desired mutations were introduced.

Growth rates and drug susceptibility testing

Growth rate experiments using wild-type and mutant strains in the exponential growth phase and drug susceptibility testing were performed as described previously.⁶

Results and discussion

Multiplicity of rRNA operons complicates the study of rRNA mutations in many bacteria. The genetically engineered strain M. smegmatis mc²155 SMR5 rRNA⁻, which contains only one functional rRNA operon, is a suitable model for studying the relationship between rRNA mutations and ribosome-targeting drug resistance.⁴,⁶,⁸,¹⁰ This M. smegmatis strain was used in this study and the single mutations A2058G, A2503U and U2504G and double mutations A2058G–A2503U and A2503U–U2504G were successfully introduced.

As observed previously,⁴,¹⁰ the A2058G alteration conferred high-level resistance to erythromycin, azithromycin and clindamycin. Compared with susceptibility to 14- and 15-membered macrolines, susceptibility to the 16-membered macrolines tylsino, spiramycin and josamycin was less affected by the A2058G mutation (Table 2).

The A2503U and U2504G mutant exhibited elevated MICs (at least 4-fold) of valnemulin, chloramphenicol, florfenicol and linezolid (Table 2). Interestingly, a recent study in M. smegmatis showed that introduction of an A2503G mutation did not affect susceptibility to valnemulin but could lead to resistance to chloramphenicol and linezolid.⁶ The A2503U mutation was found in pleuromutilin-resistant mutants of M. gallisepticum that were selected with trimethoprim and clindamycin. The A2503U mutation was detected together with other 23S rRNA mutations in resistant mutants of Staphylococcus aureus and Streptococcus pneumoniae that were selected with linezolid.¹¹,¹² These data
may explain the difference between the resistance phenotypes of the A2503U and A2503G M. smegmatis mutants. U2504 plays pivotal roles in resistance to PTC antibiotics. Moreover, U2504 is post-transcriptionally modified in many bacteria and the modification is linked to resistance to PTC antibiotics, as E. coli strains without the modification are more susceptible to tiamulin, clindamycin and linezolid.

In this study, we found that the A2503U and U2504G mutations could cause decreased susceptibility to 16-membered macrolides (Table 2). In addition, the double mutations A2058G–A2503U and A2058G–U2504G had combined effects on resistance to 16-membered macrolides. The MICs of tylosin, spiramycin and josamycin for the A2058G–A2503U mutant increased to 256, 1024 and 512 mg/L, respectively. The MICs of tylosin, spiramycin and josamycin for the A2058G–U2504G mutant increased to 64, 512 and 128 mg/L, respectively (Table 2). Sixteen-membered macrolides have a mycaminose–mycarose disaccharide at their C5 position. The side chain extends towards the PTC and participates in additional interaction with some 23S rRNA nucleotides, which are in close proximity to A2503 and U2504. The A2503U and U2504G mutations may directly or indirectly affect the conformation of the binding pocket for the disaccharide side chain and then disturb the binding of 16-membered macrolides. Moreover, the extended disaccharide side chain of 16-membered macrolides bridges the distance between A2058 and A2503/U2504 and establishes contacts with both regions. The double mutations A2058G–A2503U and A2058G–U2504G simultaneously disrupted two important interactions for drug binding and then exhibited co-effects on resistance to 16-membered macrolides.

The A2058G alteration did not affect cell growth to a major degree in M. smegmatis. The A2503U mutation led to a moderate decrease in growth rate (doubling time 4.5 h) and the largest decrease was observed for the U2504G mutant (doubling time 5.6 h). Surprisingly, the double mutations did not cause a significant decrease in growth rate compared with the corresponding single mutation. The doubling time of the A2058G–A2503U mutant was 5.4 h. The doubling time of the A2058G–U2504G mutant (4.5 h) was even less than that of the single U2504G mutant (5.6 h). One possible explanation is that the additional A2508G mutation adjusted the conformation change mediated by the U2504G mutation and compensated for the growth disadvantage of the U2504G mutation.

In summary, the mutations A2058G, A2503U and U2504G play key roles in resistance to antibiotics that target the ribosomal PTC and its associated region. The co-effects of the double mutations observed in this study reflected how the 16-membered macrolides interact with the bacterial 50S ribosomal subunit and may be helpful for the future development of improved drugs.

Acknowledgements
We are grateful to Dr Erik C. Böttger for providing strain M. smegmatis mc2155 SMR5 rrnB² and plasmid PMV361-Hyg.

Funding
This research was supported by The National Natural Science Foundation of China (grant no. 30571401) and the Joint Foundation of the NSFC-Guangdong (grant no. U0631006).

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References