Emergence of a 23S rRNA mutation in *Mycoplasma hominis* associated with a loss of the intrinsic resistance to erythromycin and azithromycin

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Objectives: *Mycoplasma hominis* is intrinsically resistant to 14- and 15-membered macrolides and to the ketolide telithromycin but is susceptible to josamycin, a 16-membered macrolide, and lincosamides. The aim of our study was to investigate the *in vitro* development of macrolide resistance in *M. hominis* and to study the impact of ribosomal mutations on MICs of various macrolides and related antibiotics.

Methods: Selection of macrolide-resistant mutants was performed by serial passages of *M. hominis* PG21 in broth medium containing subinhibitory concentrations of clindamycin, pristinamycin, quinupristin/dalfopristin and telithromycin. Stepwise selection of josamycin-resistant mutants was performed onto agar medium containing increasing inhibitory concentrations of josamycin. Resistant mutants were characterized by PCR amplification and DNA sequencing of 23S rRNA, L4 and L22 ribosomal protein genes.

Results: Various mutations in domain II or V of 23S rRNA were selected in the presence of each selector antibiotic and were associated with several resistance phenotypes. Josamycin was the sole antibiotic that selected for single amino acid changes in ribosomal proteins L4 and L22. Unexpectedly, the C2611U transition selected in the presence of clindamycin and the quinupristin/dalfopristin combination was associated with decreased MICs of erythromycin, azithromycin and telithromycin, leading to a loss of the intrinsic resistance of *M. hominis* to erythromycin and azithromycin.

Conclusions: Ribosomal mutations were associated with resistance to macrolides and related antibiotics in *M. hominis*. Some mutants showed a loss of the intrinsic resistance to erythromycin and azithromycin.

Keywords: macrolides, mutations, resistance mechanisms, *M. hominis*

Introduction

*Mycoplasma hominis* is a genital mycoplasma intrinsically resistant to 14- and 15-membered macrolides and to the ketolide telithromycin but susceptible to josamycin, a 16-membered macrolide, and lincosamides. This resistance has been mainly associated with a G2057A transition (*Escherichia coli* numbering) in domain V of 23S rRNA. Strains with acquired resistance to macrolides have rarely been described. As two copies of the 23S rRNA gene are present in *M. hominis*, both heterozygous and homozygous strains are expected. Two *in vitro*-selected mutants resistant to josamycin with A2062G or A2062T mutations in one or two alleles, respectively, have been reported. Moreover, we described two clinical isolates with a macrolide–lincosamide–streptogramin B (MLSb) resistance phenotype that harboured an A2059G substitution, alone or associated with a C2611U transition, on only the *rrnB* operon. The aim of our study was to investigate the *in vitro* development of macrolide resistance in *M. hominis* and to study the impact of ribosomal mutations on MICs of various macrolides and related antibiotics.

Materials and methods

Selection of macrolide-resistant mutants was performed, as described previously, by serial transfers of *M. hominis* in Hayflick modified broth medium supplemented with arginine, containing subinhibitory concentrations of clindamycin, pristinamycin, quinupristin/dalfopristin and telithromycin. The *M. hominis* type strain PG21 (ATCC 23114), previously used for other selection studies, was...
chosen. Twenty-five passages were performed for each selector antibiotic except for telithromycin, for which high-level resistant mutants were obtained after five passages. Finally, five consecutive subcultures in antibiotic-free medium showed that the resistance phenotype remained stable in all selected mutants. In addition, stepwise selection of josamycin-resistant mutants was performed onto Hayflick modified agar medium containing increasing inhibitory concentrations of josamycin, as described previously. Two steps were performed with josamycin concentrations at 1, 2 and 4 times the MIC for the respective parent strain. Resistant mutants were characterized by PCR amplification and DNA sequencing of three DNA fragments of the 23S rRNA gene, one in domain II (primers MH23S-17, 5'-GGGTGTACATCTTGGACATAATGG-3', and MH23S-29, 5'-GCGGCCTTGCCGATTCAG-3') and two in domain V (primers MH23S-11, MP23S-22, 5'-GCCGATACACCGGATGAAAAGCGTAC-3', and MH23S-25). When examination of the sequencing traces showed a mixture of bases at the altered residues, primers MH23S-A or MH23S-B, designed to amplify each 23S rRNA gene common to both alleles. The entire gene of protein L22 and a fragment of the L4 gene were also sequenced (primers MHL4-U, MHL4-R, MHL22-U and MHL22-R). For two clones of mutants C5 and QD6, the entire 23S rRNA, L4 and L22 genes were sequenced. Pulsed-field gel electrophoresis of SalI and BamHI-digested genomic DNA was used to confirm that mutants were derived from the parental strain. As expected, the macrorestriction digestion profile was identical to that of the parent strain (data not shown).

### Results and discussion

MICs and ribosomal mutations observed in *M. hominis* PG21 mutants are shown in Table 1 and Figure 1. The mutants selected at the 5th passage in the presence of clindamycin (mutant C5) and at the 6th and 12th passages in the presence of quinupristin/dalfopristin (QD6 and QD12) harboured a C2611U substitution in the *rrnB* operon of the 23S rRNA gene. No other alteration was found in the entirely sequenced 23S rRNA or in the L4 and L22 protein genes of mutants C5 and QD6. For both mutants, MICs of erymycin, azithromycin and telithromycin were significantly reduced with a 256-fold, 32-fold and 4-fold decrease, respectively (Table 1). Activities of other macrolides, lincosamides, streptogramins and ketolides (MLSKs) were not significantly modified. In *M. hominis*, the intrinsic resistance to 14- and 15-membered macrolides and telithromycin has been associated with a G2057A transition in domain V of 23S rRNA. This mutation could lead to a disruption of the rRNA structure with an opening of the stem preceding the single-stranded portion of the peptidyl transferase loop. In mutants C5, QD6 and QD12, the C2611U transition

<table>
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<tr>
<th>Strains</th>
<th>ERY</th>
<th>AZM</th>
<th>JOS</th>
<th>CLI</th>
<th>QUI</th>
<th>PRI</th>
<th>Q/D</th>
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<tr>
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<td>0.5</td>
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<td>64</td>
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<td>4</td>
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<sup>a</sup>Erythromycin A; AZM, azithromycin; JOS, josamycin; CLI, clindamycin; QUI, quinupristin; PRI, pristinamycin; Q/D, quinupristin/dalfopristin; TEL, telithromycin.

<sup>b</sup>E. coli numbering.

<sup>c</sup>*M. hominis* numbering.

<sup>d</sup>Clindamycin Broth selection with: for each selector antibiotic except for telithromycin (five passages). Only passages with a significant MIC increase (at least 4-fold) are presented in Table 1. For these passages, two of the five clones subcultured were studied. Only MICs of one clone are shown since no significant difference (no more than one dilution) was observed between the susceptibilities of both clones. Their nucleotide sequences were always identical.

<sup>e</sup>Agar-selected mutants are designated by a prefix corresponding to the selection step (I or II) followed by the initial of the selector josamycin (J). Only clones with significant increased MICs and ribosomal mutations are shown. Mutant IJ2 was generated from mutant IJ2 and mutant IJ4 was generated from mutant IJ4.
to an MLSB resistance phenotype with a return to high-level resistance to erythromycin (MIC 512 mg/L) (Table 1). Interestingly, we reported the same association of C2611U and A2059G substitutions on the \( rrnB \) operon, which led to the same MLSB phenotype of resistance, in a clinical isolate of \( M. \ hominis \) \(^2\). In mutant QD12, the C2611U substitution and an additional G2608C mutation were associated with a significant increase in streptogramin combination MICs, whereas erythromycin, azithromycin and telithromycin MICs remained unchanged compared with those for the QD6 mutant. To our knowledge, this mutation has never been associated with resistance to MLSKs.

In mutant T3, a G2056A transition on the \( rrnB \) operon led to the same MLSB phenotype of resistance, in a clinical isolate of \( M. \ hominis \). Moreover, in \( M. \ pneumoniae \), a base pair G2057–C2611 exists, and we described how a C2611A transversion, which disrupts this base pair, led to increased MICs of the same erythromycin, azithromycin and telithromycin antimicrobials. Consequently, the secondary structure of this portion of the peptidyltransferase loop appears to recreate a Watson–Crick base pair with the adenine at position 2057 (Figure 1c) and is associated with a loss of the intrinsic resistance to erythromycin and azithromycin and with a significant decrease in telithromycin MICs. It should be noted that in all cases the mutation of the sole \( rrnB \) operon was sufficient to significantly decrease MICs of the three antimicrobials. However, MICs remained higher than those reported for a susceptible mycoplasma, \( M. \ pneumoniae \). Moreover, in \( M. \ pneumoniae \), a base pair G2057–C2611 exists, and we described how a C2611A transversion, which disrupts this base pair, led to increased MICs of the same erythromycin, azithromycin and telithromycin antimicrobials. Consequently, the secondary structure of this portion of the peptidyltransferase loop appears to be strongly involved in the susceptibility of \( M. \ hominis \) and \( M. \ pneumoniae \) to these three MLSKs.

In mutant C10, an additional A2059G on the \( rrnB \) operon led to an MLSB resistance phenotype with a return to high-level resistance to erythromycin (MIC 512 mg/L) (Table 1). Interestingly, we reported the same association of C2611U and A2059G substitutions on the \( rrnB \) operon, which led to the same MLSB phenotype of resistance, in a clinical isolate of \( M. \ hominis \) \(^2\). In mutant QD12, the C2611U substitution and an additional G2608C mutation were associated with a significant increase in streptogramin combination MICs, whereas erythromycin, azithromycin and telithromycin MICs remained unchanged compared with those for the QD6 mutant. To our knowledge, this mutation has never been associated with resistance to MLSKs.

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At the sixth passage, mutants selected in the presence of pristinamycin harboured a C2586U substitution on the \textit{rrnB} operon, which was associated with a 4-fold increased MIC of both streptogramin combinations. To date, mutations at position 2586 have never been associated with MLSK resistance, but the crystal structure analysis of the large subunit of \textit{Deinococcus radiodurans} complexed with the quinupristin/dalfopristin combination revealed that a hydrogen bond exists between quinupristin and nucleotide 2586 (\textit{E. coli} numbering). However, the MIC of quinupristin was not significantly increased in mutant P6. In addition, a hydrogen bond also exists between base 2586 and azithromycin complexed to the 50S subunit of \textit{D. radiodurans}, but the azithromycin MIC was not affected in mutant P6. In mutant P25, an additional G792A mutation in domain II of 23S rRNA, also on operon \textit{rrnB}, led to resistance to both streptogramin combinations and to increased MICs of josamycin and streptogramin combinations. In the L4 protein, a histidine to leucine substitution at position 184 (mutants IJ4 and IIJ4) was associated with increased MICs of azithromycin and telithromycin, leading to a loss of intrinsic resistance of \textit{M. hominis} to azithromycin and telithromycin. Reconstruction of this mutation into a wild-type strain of \textit{M. hominis} would prove its involvement in the phenotype observed. However, only a few genetic tools are available in mycoplasmas, especially in human species, and, at this time, directed mutagenesis through homologous recombination has not been successfully applied to \textit{M. hominis}. Development of genetic tools for \textit{M. hominis} is required to demonstrate the exact significance of the mutation observed.

**References**


