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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.[1,2] This resistance has been mainly associated with a G2057A transition (*Escherichia coli* numbering) in domain V of 23S rRNA.[2] Strains with acquired resistance to macrolides have rarely been described. As two copies of the 23S rRNA gene are present in *M. hominis*,[2] 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.[3] Moreover, we described two clinical isolates with a macrolide–lincosamide–streptogramin B (MLS_B) resistance phenotype that harboured an A2059G substitution, alone or associated with a C2611U transition, on only the *rrnB* operon.[2] 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,[4] 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,[3] was

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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.[5] 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,[4] one in domain II (primers MH23S-17, 5′-GCGTACATCTTGCAGAATGG-3′, and MH23S-29, 5′-CGCCGCCATTCCATATTCAG-3′) and two in domain V (primers MH23S-11, MP23S-22,[4] MH23S-9, 5′-GCTCAACGGATAAAAGC-TAC-3′, and MH23S-25).[2] When examination of the sequencing traces showed a mixture of bases at the altered residues, primers MH23S-A or MH23S-B,[2] designed to amplify each 23S rRNA gene independently, were used for amplification with primer MH23S-17 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).[2] For two clones of mutants C5 and QD6, the entire 23S rRNA, L4 and L22 genes were sequenced. Pulsed-field gel electrophoresis of *Sal*I and *Bam*HI-digested genomic DNA was used to confirm that mutants were derived from the parental strain.[6] 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 erythromycin, 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.[2] 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 1. MICs and ribosomal mutations observed in *M. hominis* PG21 mutants selected by serial passages in subinhibitory concentrations of clindamycin, pristinamycin, quinupristin/dalfopristin and telithromycin and by stepwise exposure to inhibitory concentrations of josamycin

Strains	MIC (mg/L)[a]								23S rRNA[b] (heterozygosity *rrnA*:*rrnB*) and riboprotein[c] mutations
	ERY	AZM	JOS	CLI	QUI	PRI	Q/D	TEL	
M. hominis									
PG21	512	64	0.5	0.25	64	1	2	32	none
Clindamycin Broth selection with:[d]									
C5	2	2	0.5	0.5	128	2	4	8	C2611U (1C:1U)
C10	512	512	512	64	64	1	4	512	C2611U (1C:1U) + A2059G (1A:1G)
Pristinamycin									
P6	512	64	0.5	0.25	128	4	8	64	C2586U (1C:1U)
P25	512	128	4	0.5	128	64	64	128	C2586U (1C:1U) + G792A (1G:1A)
Quinupristin/dalfopristin									
QD6	2	2	0.5	0.5	128	2	4	8	C2611U (1C:1U)
QD12	1	2	1	0.5	128	32	64	8	C2611U (1C:1U) + G2608C (1G:1C)
Telithromycin									
T3	512	256	1	0.25	64	2	4	256	G2056A (1G:1A)
Agar selection with josamycin[e]									
IJ2	512	64	4	1	128	1	2	16	G2576U (1U:1G)
IIJ2	512	512	8	2	128	2	2	256	G2576U (1U:1G) + G2056A (1A:1G)
IJ1	512	256	16	4	64	0.5	2	128	L22, R97K
IJ4	512	128	16	0.5	128	4	8	64	L4, H184L
IIJ4	512	512	512	0.5	128	64	64	256	L4, H184L + G792A (1A:1G) + A2062C (1A:1C)

[a]ERY, erythromycin A; AZM, azithromycin; JOS, josamycin; CLI, clindamycin; QUI, quinupristin; PRI, pristinamycin; Q/D, quinupristin/dalfopristin; TEL, telithromycin.
[b]*E. coli* numbering.
[c]*M. hominis* numbering.
[d]*M. hominis* PG21 is the parental strain. Mutants selected in broth medium are designated by the initial of the selector antibiotic followed by the passage number. Twenty-five passages were performed 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.
[e]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 IIJ2 was generated from mutant IJ2 and mutant IIJ4 was generated from mutant IJ4.

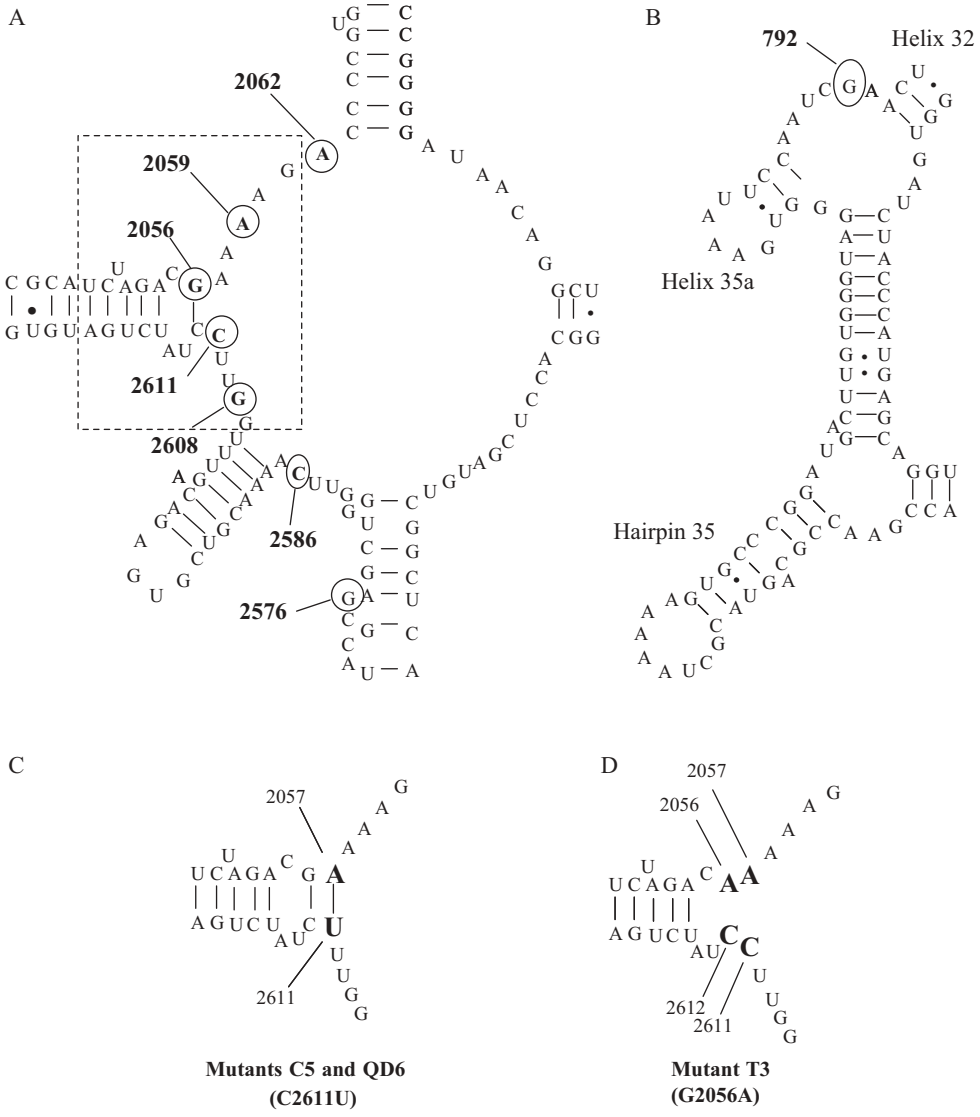

Figure 1. Secondary structure of the peptidyl transferase loop in domain V of 23S rRNA (a) and part of domain II (b) of *M. hominis* PG21. The circled nucleotides indicate the position of mutations encountered in mutants selected in this study. The nucleotides are numbered on the basis of the *E. coli* sequence. (c) Structure of the framed region of mutants C5 and QD6. The C2611U substitution recreates a base pair between nucleotides 2057 and 2611. (d) Structure of the framed region of mutant T3. The G2056A transition disrupts the 2056–2612 base pair.

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, *Mycoplasma pneumoniae*.[1] 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 seems 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 MLS_B 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 MLS_B 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 was selected at the third passage in the presence of telithromycin. This mutation was associated with 4- and 8-fold increased MICs of azithromycin and telithromycin, respectively. It should be noted that this G2056A substitution disrupts another base pair in *M. hominis* (G2056–C2612) and could induce a more open conformation of the peptidyl transferase loop (Figure 1d).

At the sixth passage, mutants selected in the presence of pristinamycin harboured a C2586U substitution on the *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 *Deinococcus radiodurans* complexed with the quinupristin/dalfopristin combination revealed that a hydrogen bond exists between quinupristin and nucleotide 2586 (*E. coli* numbering).[7] 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 *D. radiodurans*,[8] 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 *rrnB*, led to resistance to both streptogramin combinations and to increased MICs of josamycin. The MIC of telithromycin was also 4-fold higher than that for the parental strain. Nucleotide 792 is located in the loop connecting helices 32 and 35a (Figure 1b), in close proximity to the base 790, which is involved in the binding of ketolides.[8,9] This substitution was also observed on operon *rrnA* of the josamycin-resistant mutant IIJ4 obtained by agar selection (Table 1).

Five josamycin-resistant mutants were obtained by agar stepwise selection. Mutant IJ2 harboured a G2576U substitution on the *rrnA* operon. This substitution has been associated with resistance to linezolid in *Enterococcus* spp. and *Staphylococcus aureus*.[10] However, the MIC of linezolid for the IJ2 mutant was identical to that for the parental strain PG21 (MIC 8 mg/L). Mutant IIJ2, generated from IJ2, presented an additional G2056A mutation also on the *rrnA* operon, which was associated with significantly increased MICs of azithromycin and telithromycin. Interestingly, the same G2056A substitution is carried by operon *rrnB* in mutant T3 and by operon *rrnA* in mutant IIJ2 and is associated with the same phenotype of resistance (Table 1). To date, this is the first description of macrolide-resistant mutants of *M. hominis* harbouring mutations on the *rrnA* operon. It should be noted that mutations on this operon were selected only by stepwise selection on agar medium, but this observation should be confirmed.

For the first time in *M. hominis*, mutations in ribosomal proteins L4 and L22 were observed in macrolide-resistant mutants. In the L22 protein, an arginine to lysine substitution at position 97 (mutant IJ1, *M. hominis* numbering) was associated with an MLS_B phenotype. In the L4 protein, a histidine to leucine mutation at position 184 (mutants IJ4 and IIJ4) was associated with increased MICs of josamycin and streptogramin combinations and was previously reported in an *in vitro*-resistant mutant of *M. pneumoniae* (H70L, *M. pneumoniae* numbering).[4] Mutant IIJ4 harboured three mutations in L4 and 23S rRNA domains II and V that led to high-level resistance to macrolides, streptogramin combinations and telithromycin (Table 1). Only clindamycin remained active against this mutant. Interestingly, alterations at position 2062 were previously associated with resistance to 16-membered macrolides and streptogramin combinations in *M. hominis* and *M. pneumoniae*.[3,4] Moreover, the crystal structure of the 50S ribosomal subunit from *D. radiodurans* complexed with the quinupristin/dalfopristin combination indicates that both compounds have contacts with nucleotide 2062.[7]

To summarize, a diversity of mutations can be selected *in vitro* with different macrolides and related antibiotics. Whereas most mutations were associated with increased MICs, the C2611U transition was unexpectedly associated with decreased MICs of erythromycin, azithromycin and telithromycin, leading to a loss of intrinsic resistance of *M. hominis* to erythromycin and azithromycin. Reconstruction of this mutation into a wild-type strain of *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 *M. hominis*. Development of genetic tools for *M. hominis* is required to demonstrate the exact significance of the mutation observed.

Transparency declarations

None to declare.

References

1. Bébéar CM, Kempf I. Antimicrobial therapy and antimicrobial resistance. In: Blanchard A, Browning GF, eds. *Mycoplasmas: Molecular Biology, Pathogenicity and Strategies for Control*. Norfolk: Horizon Bioscience, 2005; 535–68.

2. Pereyre S, Gonzalez P, de Barbeyrac B *et al*. Mutations in 23S rRNA account for intrinsic resistance to macrolides in *Mycoplasma hominis* and *Mycoplasma fermentans* and for acquired resistance to macrolides in *M. hominis*. *Antimicrob Agents Chemother* 2002; **46**: 3142–50.

3. Furneri PM, Rappazzo G, Musumarra MP *et al*. Two new point mutations at A2062 associated with resistance to 16-membered macrolide antibiotics in mutant strains of *Mycoplasma hominis*. *Antimicrob Agents Chemother* 2001; **45**: 2958–60.

4. Pereyre S, Guyot C, Renaudin H *et al*. *In vitro* selection and characterization of resistance to macrolides and related antibiotics in *Mycoplasma pneumoniae*. *Antimicrob Agents Chemother* 2004; **48**: 460–5.

5. Bébéar CM, Renaudin H, Charron A *et al*. Alterations in topoisomerase IV and DNA gyrase in quinolone-resistant mutants of *Mycoplasma hominis* obtained *in vitro*. *Antimicrob Agents Chemother* 1998; **42**: 2304–11.

6. Bébéar CM, Charron A, Bové JM *et al*. Cloning and nucleotide sequences of the topoisomerase IV *parC* and *parE* genes of *Mycoplasma hominis*. *Antimicrob Agents Chemother* 1998; **42**: 2024–31.

7. Harm JM, Schlunzen F, Fucini P *et al*. Alterations at the peptidyl transferase centre of the ribosome induced by the synergistic action of the streptogramins dalfopristin and quinupristin. *BMC Biol* 2004; **2**: 4.

8. Schlunzen F, Harms JM, Franceschi F *et al*. Structural basis for the antibiotic activity of ketolides and azalides. *Structure* 2003; **11**: 329–38.

9. Berisio R, Harms J, Schluenzen F *et al*. Structural insight into the antibiotic action of telithromycin against resistant mutants. *J Bacteriol* 2003; **185**: 4276–9.

10. Marshall SH, Donskey CJ, Hutton-Thomas R *et al*. Gene dosage and linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrob Agents Chemother* 2002; **46**: 3334–6.