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Increased Expression of Two Multidrug Transporter-Like Genes Is Associated with Ethidium Bromide and Ciprofloxacin Resistance in *Mycoplasma hominis*

S. Raherison, P. Gonzalez, H. Renaudin, A. Charron, C. Bébéar, and C. M. Bébéar*

Laboratoire de Bactériologie, Université Victor Segalen Bordeaux 2, Bordeaux Cedex, France

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Two genes, *md1* and *md2*, coding for multidrug resistance ATP-binding cassette transporters were identified in *Mycoplasma hominis* PG21. Expression of these two genes, quantified by quantitative competitive reverse transcription-PCR, was significantly increased in ethidium bromide-resistant strains of *M. hominis* compared to that in *M. hominis* PG21.

Mycoplasma hominis is a cause of human urogenital tract infections for the treatment of which fluoroquinolones represent an efficient antimicrobial class. Three mechanisms of bacterial resistance to fluoroquinolones have been described, target-related mechanisms, by either alteration or protection, and active efflux (7). Active efflux of fluoroquinolones is mediated by endogenous multidrug resistance (MDR) efflux pumps, increased expression of which can develop an MDR phenotype (15). In *M. hominis*, resistance by target alteration has already been described in vivo (2) and in vitro (1). Furthermore, we recently reported an active efflux system, possibly an ATP-binding cassette (ABC)-type efflux pump, in ethidium bromide (EtBr)-selected strains of *M. hominis* showing an MDR phenotype with increased MICs of ciprofloxacin and EtBr (16).

Few bacterial ABC MDR efflux systems have been characterized; all are homologous to the known human P glycoprotein LmrA in *Lactococcus lactis* (19), MsbA in *Escherichia coli* (3), HorA in *Lactobacillus brevis* (17), VcaM in *Vibrio cholerae* (8), and more recently the heterodimeric ABC transporter EfrAB in *Enterococcus faecalis* (10). Mycoplasmal genome sequencing revealed the presence of two adjacent ABC-type genes identified as putative MDR genes, *mg014* and *mg015* in *Mycoplasma genitalium* and *pmd1* and *msbA* in *Mycoplasma pneumoniae* (13, 18). To determine the genetic support of the ciprofloxacin and EtBr active efflux identified in *M. hominis*, we searched for homologous genes in the *M. hominis* genome.

A consensus primer, MD2-1 (5′-GGTCCTACAGGAACT GGAAAA-3′), located in the Walker A motif of the ATP-binding domain and a degenerated primer, MD2R (5′-ATAA TYTCHTCWTYWGTDGCAT-3′), located downstream of this motif just before the ABC signature were deduced from the alignment of MDR-like genes of *M. genitalium* (5) and *M. pneumoniae* (6). PCR amplification of the genomic DNA from the *M. hominis* PG21 reference strain with primers MD2-1 and MD2R led to a 224-bp DNA fragment showing homology with the *mg014* gene of *M. genitalium*. Two recombinant plasmids selected by colony hybridization with the radiolabeled 224-bp DNA fragment were obtained from two HindIII and XbaI genomic libraries of *M. hominis* PG21. Inserts of these two recombinant plasmids were sequenced by primer walking. The 10,616-bp DNA sequence obtained was shown to contain eight putative open reading frames, two of which, E and F, were assigned as MDR-like genes in *M. hominis* and named *md1* and *md2*, respectively. Analysis of the upstream region of gene *md1* revealed a putative promoter and a consensus Shine-Dalgarno sequence located upstream of the ATG start codon. The TAG stop codon of the *md1* gene is preceded by the ATG start codon of *md2*, and the two genes overlap by 8 nucleotides. A short stem-loop structure, followed by a run of T residues corresponding to a rho-independent transcription terminator frequently found in mollicutes (11), was found only downstream of the *md2* gene stop codon. The predicted MD1 and MD2 proteins contained 607 and 625 amino acids, respectively, corresponding to calculated molecular masses of 68 and 70 kDa. Kyte-Doolittle hydropathy plots detected one hydrophilic carboxyl-terminal domain and one hydrophobic amino-terminal domain in both proteins. The hydrophobic domain contained six potential transmembrane segments (TMS) as described for LmrA in *L. lactis* (19), HorA in *L. brevis* (17), and other bacterial ABC MDR pumps (3, 8, 10). An ATP-binding domain was found in the carboxyl-terminal domain of both

TABLE 1. Percentages of identity and similarity between the MD1 and MD2 proteins of *M. hominis* and other ABC-type MDR transporters

Transporter	Organism	Identity similarity (%)		Reference
		MD1	MD2	
MD1	*M. hominis*	100, 100	27.3, 68.3	This study
MD2	*M. hominis*	27.3, 68.3	100, 100	This study
EfrA	*E. faecalis*	29, 71.2	27.8, 67.8	12
EfrB	*E. faecalis*	24.2, 66.8	34.4, 72.8	12
HorA	*L. brevis*	13.9, 57.2	28, 67	17
LmrA	*L. lactis*	13.7, 56.9	26.6, 69.1	19
MsbA	*E. coli*	23.6, 65.9	29.1, 66.5	9
VceM	*V. cholerae*	21.8, 61.3	22.4, 62	8
MDR1N[a]	*Homo sapiens*	25.2, 68.3	26.9, 66.1	4
MDR1C[a]	*Homo sapiens*	23.8, 65.5	25.2, 67	4

[a] MDR1C and MDR1N correspond to the carboxy-terminal and amino-terminal halves of the human P glycoprotein, respectively.

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France. Phone: (33) 5.57.57.16.25. Fax: (33) 5.56.93.29.40. E-mail: cecile.bebear@u-bordeaux2.fr.

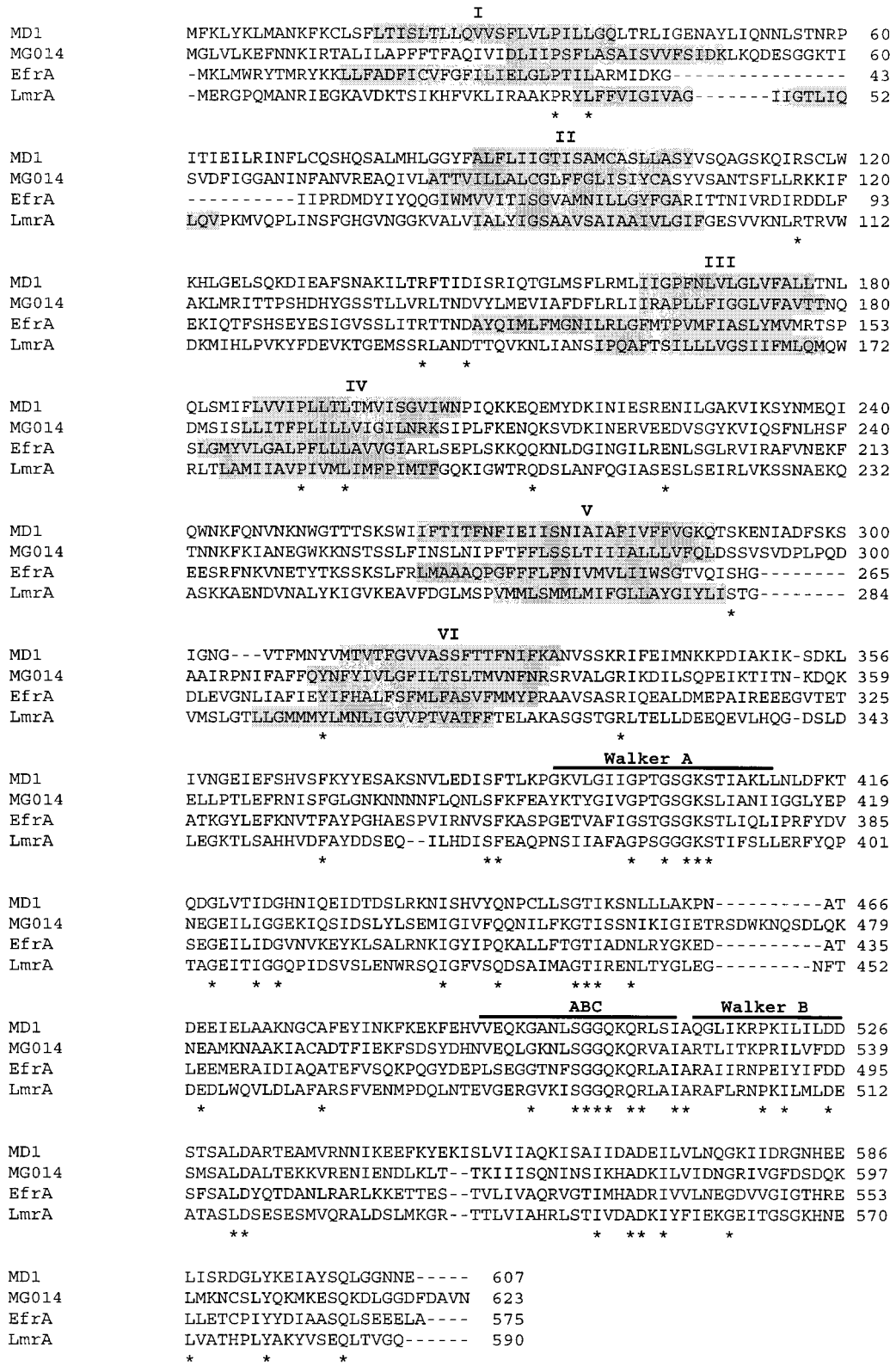

FIG. 1. ClustalW alignment of the deduced amino acid sequences of MD1 from *M. hominis* (GenBank accession no. AY169817), MG014 from *M. genitalium* (5), EfrA from *E. faecalis* (12), and LmrA from *L. lactis* (19). Asterisks indicate identical residues. Shading indicates the putative transmembrane α-helices predicted by TMpred from the INFOBIOGEN website. The ABC signature sequence and Walker A and B motifs are indicated by horizontal lines.

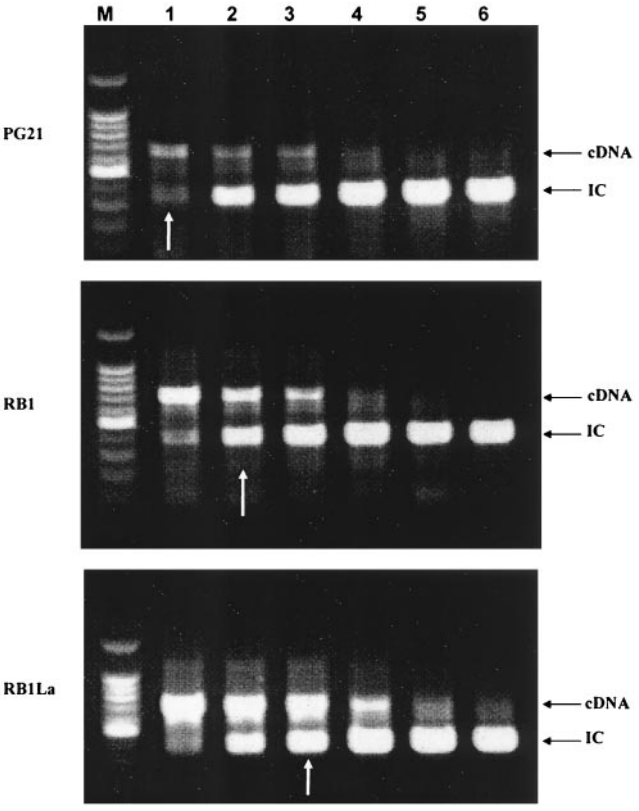

FIG. 2. Quantitative competitive RT-PCR of the *md1* gene in *M. hominis* strains PG21, RB1, and RB1La. White arrows indicate the cDNA quantity detected for each strain compared to the internal control (IC) range. Lanes: M, 100-bp molecular mass marker; 1, 0.03 pg; 2, 0.06 pg; 3, 0.2 pg; 4, 0.5 pg; 5, 1 pg; 6, 2 pg.

proteins, including the characteristic Walker A and B motifs and the ABC signature sequence (18). MD1 and MD2 showed 27.3% sequence identity and 68.3% similarity. ClustalW comparison of the MD1 and MD2 proteins with the other ABC-type MDR proteins identified in bacteria and the two halves of the human P glycoprotein is summarized Table 1. Proteins MD1 and MD2 showed the best levels of identity and similarity with *E. faecalis* MDR proteins EfrA and EfrB, respectively. It should be noted that the *efrA* and *efrB* genes seem to be organized in an operon like *md1* and *md2*, with the two genes overlapping and being followed by a transcription terminator-like sequence (10). The sequence alignments of the MD1, MG014, EfrA, and LmrA proteins are shown Fig. 1. TMS prediction with the TMpred program indicated that the six TMSs of the MD1 protein were at positions similar to those of the other ABC MDR pumps (18, 19). The Walker A and B motifs and the ABC signature sequence were conserved in all four proteins (Fig. 1).

Expression of *md1* and *md2* in *M. hominis* PG21 and the MDR phenotype strains RB1 and RB1La selected on EtBr (16) was studied and quantified by quantitative competitive reverse transcription (RT)-PCR (14) (Fig. 2). RNAs were isolated from mycoplasma cultures in the exponential growth phase with the High Pure RNA isolated kit (Roche Diagnostics GmbH) and quantified by spectrophotometry. mRNAs were reverse transcribed into cDNA with the Enhanced Avian RT-PCR kit (Sigma). Internal competitor (IC) DNAs were generated by PCR amplification from *M. hominis* PG21 with primers a_{20}MD1 (5′-TGTCAAAGCCATCAGAGTGC G-3′) and $b_{20}c_{20}$MD1 (5′-AATGAAGAAGCAACAGCTCCT TTGCTTGTTGTGGTTCCTC-3′) for the *md1* IC and primers a_{20}MD2 (5′-TAGTGCTTTAACATCGCTTGG-3′) and $b_{20}c_{20}$MD2 (5′-AGGTCCTACAATAGCAAACACCAACGC AAATGCTCCGCCAAC-3′) for the *md2* IC. Each IC was added at concentrations of 0.03 to 2 pg to a constant amount of cDNA. The PCR amplification was performed on the IC DNA-cDNA mixture with primers a_{20}MD1 and c_{20}MD1 (5′-AATGAAGAAGCAACAGCTCC-3′) for *md1* and primers a_{20}MD2 and c_{20}MD2 (5′-AGGTCCTACAATAGCAAACAC-3′) for *md2*. After agarose gel electrophoresis, EtBr-stained PCR products were visually quantified with a UV lamp by comparing the relative amounts of the two products, which are distinct in size. For *md1* expression, strain PG21 expressed 0.03 pg of mRNA whereas the RB1 and RB1La strains expressed 0.07 and 0.2 pg of mRNA, respectively, i.e., two- and sevenfold more than the PG21 strain (Fig. 2). In the same way, expression of *md2* was 3- and 10-fold increased for RB1 and RB1La, respectively, compared to that of PG21 (data not shown). These results indicated constitutive expression of both genes in control strain PG21 and overexpression in the MDR phenotype strains. The most EtBr-resistant strain, RB1La, which had the lowest CIP and EtBr uptake levels (16), also had the highest level of *md1* and *md2* expression, strengthening the association of these genes with the MDR phenotype observed in *M. hominis*.

To explain this overexpression, we looked for mutations in the putative *md1* promoter region of the RB1 and RB1La strains. However, no point mutation was detected in this region. In the same way, no mutation was found within the *md1* and *md2* sequences or in the 5-kbp region upstream of *md1*. No gene homologous to transcriptional regulator families regulating the expression of bacterial MDR pumps has been found in the mycoplasmal genomes completely sequenced. The mechanism of regulation of the expression of genes *md1* and *md2* remains to be determined. Genetic inactivation of these two genes in EtBr-resistant strains would prove their involvement in the MDR efflux of *M. hominis*. However, gene disruption through homologous recombination has not been successfully applied to *M. hominis*. Therefore, direct evidence of the role of the MDR genes identified in this study in *M. hominis* would benefit from the development of genetic tools for this microorganism.

Nucleotide sequence accession number. The nucleotide sequence data reported for *M. hominis* have been submitted to the GenBank database and assigned accession no. AY169817.

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