Journal of Antimicrobial Chemotherapy (2001) **47**, 651–654

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Decreased susceptibility to azithromycin and erythromycin mediated by a novel *mtr*(R) promoter mutation in *Neisseria gonorrhoeae*

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During a screen of *Neisseria gonorrhoeae* clinical isolates obtained in Uruguay for susceptibility to azithromycin, we noticed that approximately 10% of the strains examined displayed decreased susceptibility to azithromycin and erythromycin due to the *mtr*(CDE)-encoded efflux pump system, but remained susceptible to Triton X-100. We now report that the *mtr*(R) promoter region of one of these isolates contains a dinucleotide insertion (TT) that mediates this resistance phenotype.

Introduction

Azithromycin has been proposed in Uruguay for the treatment of uncomplicated gonococcal infections because of the prevalence of strains of *Neisseria gonorrhoeae* with clinically significant levels of resistance to penicillin and tetracycline. Antimicrobial susceptibility surveillance studies demonstrated that isolates with decreased azithromycin susceptibility obtained from infected individuals are prevalent.[1]

The *mtr*(CDE)-encoded efflux pump of *N. gonorrhoeae* mediates an energy-dependent efflux process of structurally diverse hydrophobic agents (HAs) in gonococci.[2–6] The expression of this system is negatively regulated by the product of the adjacent but divergent *mtr*(R) gene.[3,5] The molecular basis for azithromycin resistance in gonococci mediated by the *mtr*(CDE)-encoded efflux pump has been described recently.[1] We reported previously that a single T:A bp deletion within the *mtr*(R) promoter region, which would decrease the level of expression of *mtr*(R), and a missense mutation at codon 45 in the *mtr*(R)-coding region, frequently occur in strains of gonococci isolated from our patient population. The presence of such mutations explains the decreased azithromycin susceptibility property in clinical isolates with a multiple resistance phenotype (e.g. expression of cross-resistance to erythromycin, Triton X-100 and crystal violet).[1]

During the course of our studies, we identified another group of gonococcal isolates that expressed decreased susceptibility to azithromycin and erythromycin because of the *mtr*(CDE)-encoded efflux pump. Interestingly, these strains remained susceptible to Triton X-100 even though *mtr*(R) mutations typically result in high levels of gonococcal resistance to Triton X-100.[2,5] DNA sequence analysis of the *mtr*(R) promoter region of one such strain (9604) revealed that it contained a novel mutation, a double TT insertion within the 13 bp inverted repeated sequence. This dinucleotide insertion increased the spacing between the –10 and –35 hexamers of the *mtr*(R) promoter from an optimal 17 nucleotides to an unfavourable 19 nucleotides.[1] We now report that this mutation can decrease *mtr*(R) gene expression in gonococci, resulting in enhanced *mtr*(CDE) gene expression and decreased bacterial susceptibility to azithromycin and erythromycin without changing susceptibility to other HAs.

Materials and methods

Strains of N. gonorrhoeae used and growth conditions

Clinical isolate 9604 was recovered from a male patient with urethritis in 1996. Strain FA19 is wild-type with respect to the *mtr*(CDE)-encoded efflux pump system and is sensitive to HAs. Its isogenic transformant strain KH15

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is hyper-resistant to HAs and contains a single base pair deletion in a 13 bp inverted repeat sequence within the promoter region of the *mtr*(R) gene (Table).[2,3] All strains were grown on GC agar base containing glucose and iron supplements at 37°C in 3.8% (v/v) CO_2.

Transformation studies and susceptibility testing

Piliated colonies of strain FA19 were transformed to increased resistance to erythromycin with PCR products (see below) of the *mtr*(R) gene from strain 9604, as described previously.[2] The susceptibilities of the transformants to azithromycin, erythromycin and Triton X-100, were determined along with the parental and the donor strains by the agar dilution method.[1]

PCR amplification and DNA sequencing studies

Chromosomal DNA from strain 9604 was used in PCRs with oligonucleotide primers RPMAL#2 and KH9#3 to amplify the complete *mtr*(R) gene, which included the promoter region.[6] A PCR product that encompassed the *mtr*(R) promoter region and the first 200 bp of the *mtr*(R) coding region was obtained with oligonucleotide primers KH9#3 and KH9#1; the latter primer anneals in the *mtr*(R) coding region.[6] Purified PCR products were used for the transformation experiments. Automated DNA sequencing on purified PCR products was performed on both DNA strands at the Emory University DNA Sequencing Core Facility.

RNA preparation and RT–PCR

Total RNA from strains FA19, KH15 and LZ6 [a transformant of strain FA19 obtained by transformation with *mtr*(R) gene from strain 9604] was prepared as described previously.[7] cDNA was synthesized from 500 ng of total RNA. RT–PCRs for the *rmp* (reduction modifiable protein) gene, which is not controlled by MtrR,[3] were performed as an internal control to assure that equal amounts of RNA were used in the reaction. RNA was reverse transcribed with Superscript II RNase H^- reverse transcriptase (Gibco-BRL, Grand Island, NY, USA) using the following primers: rmp-2 (5′-GTG TTG GTG ATG ATT GCG TGC C-3′), RPMAL#2,[6] mtrD-5 (5′-CAA GGA ACA CGG ACA AGA GCG-3′), DSD-5 (5′-CGG CCA TAA ACA ATG CCC GGC-3′) for *rmp*, *mtr*(R), *mtr*(D) and *mtr*(F) genes, respectively.

Results and discussion

DNA sequence analysis of the complete *mtr*(R) gene of strain 9604 revealed a wild-type helix–turn–helix (HTH) motif of the MtrR regulatory protein (data not shown) that is necessary for MtrR repression of *mtr*(CDE) expression.[2,3,6] However, downstream of the HTH region we detected the presence of two missense mutations that would cause amino acid replacements at positions 86 (Thr→Ala) and 105 (His→Tyr) in MtrR. In order to test whether the dinucleotide (TT) insertion in the *mtr*(R) promoter region was sufficient to cause decreased susceptibility of gonococci to erythromycin and azithromycin, we used PCR products that encompassed either the entire *mtr*(R) gene (including the promoter region) or the promoter region and the first 200 bp of the *mtr*(R) coding region in transformation experiments. We found that both PCR products could transform strain FA19 for decreased susceptibility to erythromycin. However, these transformants did not display decreased susceptibility to TX-100 (Table). The presence of the dinucleotide (TT) insertion in the *mtr*(R) promoter was confirmed in representative transformants

Table. *mtr*(R) promoter mutations and antibiotic susceptibility of gonococcal strains

		MIC[a] (mg/L)		
Strain	*mtr*(R) promoter region	azithromycin	erythromycin	Triton X-100
9604	TT insertion	0.5	2	250
FA19	wild-type	0.063	0.25	125
LZ6[b]	TT insertion	0.25	2	125
LZ7[b]	TT insertion	ND[c]	2	125
KH 15[d]	1 bp deletion	0.5	2	>16 000

[a]A two-fold dilution diference in MIC values is not considered to be significant.
[b]LZ6 and LZ7 are transformants of strain FA19 obtained by transformation with PCR products of the complete *mtr*(R) gene (LZ6) or the *mtr*(R) promoter region with 200 bp of the 5′-end of the *mtr*(R) coding region (LZ7) from strain 9604.
[c]ND, not determined.
[d]Strain KH15 is a transformant of FA19 that contains a base pair deletion in the 13 bp inverted repeat located within the *mtr*(R) promoter region.[2,3]

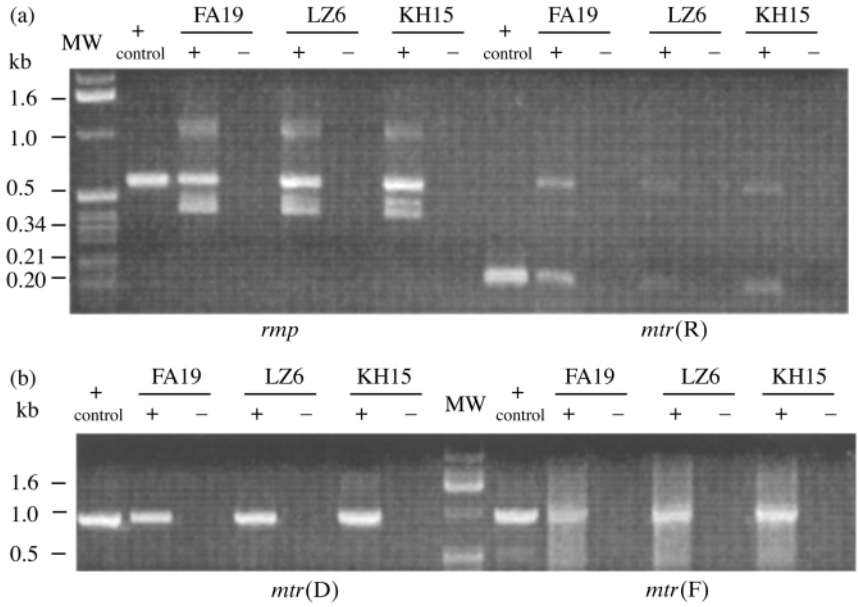

Figure. Detection of (a) *rmp* and *mtr*(R), and (b) *mtr*(D) and *mtr*(F) transcripts in isogenic strains of *N. gonorrhoeae* FA19, LZ6 and KH15 by RT–PCR. Lanes: + controls, PCR amplification products from DNA of strain FA19; –, RT–PCRs in the absence of reverse transcriptase to confirm that RNA preparations were free of contaminating DNA; MW, molecular weight marker.

by DNA sequencing (data not presented), indicating that it was sufficient for mediating the resistance property of strain 9604. Importantly, the level of azithromycin and erythromycin resistance in transformant strain LZ6 was similar to that of the donor strain 9604. In a previous study, we demonstrated that a representative transformant of isolate 9604 bearing an inactivated *mtr*(C) gene displayed significantly enhanced susceptibility to azithromycin, erythromycin and Triton X-100.[1] Thus, the efflux pump in this strain can recognize all three agents but the relevant *mtr*(R) mutation provides for enhanced resistance only to the macrolide antibiotics.

The dinucleotide insertion (TT) within the *mtr*(R) promoter region would increase the spacing between the –10 and –35 promoter hexamers. We hypothesized that this unfavourable distance would reduce the binding of RNA polymerase, and thereby repress and/or reduce transcription of the *mtr*(R) gene. Accordingly, we compared levels of the *mtr*(R) transcript by RT–PCR in RNA preparations from isogenic transformants of strain FA19 that contained the double TT insertion (strain LZ6) or a single bp deletion in the *mtr*(R) promoter (strain KH15).[2] Our results showed that both strains had a decreased level of the *mtr*(R) transcript compared with parental strain FA19 (Figure, a). We next examined whether the decreased transcription of *mtr*(R) observed with strain LZ6 would result in enhanced transcription of the *mtr* efflux pump protein encoding genes, as was observed previously for transcription of *mtr*(CDE) in strain KH15.[3] For this purpose we examined the level of the *mtr*(D) transcript, which encodes the cytoplasmic membrane transporter protein (MtrD) of the *mtr* efflux pump.[4] We found that in both strains KH15 and LZ6, the level of the *mtr*(D) transcript was elevated compared with that of strain FA19. Recently, we have identified a fourth protein that appears to be involved in expression of high-level HA resistance mediated by the *mtr* efflux pump, which is encoded by a gene [*mtr*(F)] downstream of *mtr*(R) (W. Veal & W. M. Shafer, manuscript in preparation). As with transcription of *mtr*(D), the level of *mtr*(F) transcription was increased in strains KH15 and LZ6 compared with parental strain FA19 (Figure, b).

The levels of the *mtr*(D) and *mtr*(F) transcripts in strains KH15 and LZ6 differed in that those produced by strain FA19 were at a reproducibly higher level. This difference in transcript levels may explain why strain KH15 displays a higher level of resistance to Triton X-100, a non-ionic detergent that is a substrate for the efflux pump. Presumably, the level of *mtr*(CDE) and *mtr*(F) expression imparted by the TT insertion in the *mtr*(R) promoter is sufficient to give enhanced resistance to macrolides but not Triton X-100. In this respect, we have noticed that 10% of the clinical isolates expressing decreased susceptibility to azithromycin (MIC 0.5 mg/L) and erythromycin (MIC 2 mg/L) remain susceptible to Triton X-100 (MIC 125 mg/L). DNA sequencing analysis of one additional strain expressing this phenotype revealed the presence of the TT insertion (data not presented). Our findings suggest that screening of gonococcal clinical isolates for the *mtr* phenotype based on decreased susceptibility to Triton X-100 may miss strains with mutations such as the TT insertion in the *mtr*(R) promoter, which produces decreased susceptibility to macrolide antibiotics alone.

Acknowledgements

We thank Lane Pucko for help in manuscript preparation and Jacqueline Balthazar for excellent technical assistance. This work was supported by funds from PEDECIBA and CSIC (Uruguay) and by NIH grant AI-21150 to W.M.S. L.Z. is the recipient of the International Fellowship for Latin American Students from the American Society for Microbiology. W.M.S. is the recipient of a Senior Research Career Scientist Award from the VA Medical Research Service.

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Received 6 October 2000; returned 29 November 2000; revised 2 January 2001; accepted 19 January 2001