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Mutations in a 23S rRNA Gene of *Chlamydia trachomatis* Associated with Resistance to Macrolides

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For six clinical isolates of *Chlamydia trachomatis*, in vitro susceptibility to erythromycin, azithromycin, and josamycin has been determined. Four isolates were resistant to all the antibiotics and had the mutations A2058C and T2611C (*Escherichia coli* numbering) in the 23S rRNA gene. All the isolates had mixed populations of bacteria that did and did not carry 23S rRNA gene mutations.

Chlamydia trachomatis is an obligate intracellular parasite that causes a wide range of inflammations of the urogenital tract. Clinical isolates showing resistance to azithromycin and associated with a recurrent infection have been described previously (19). The resistance of various microorganisms to macrolides is often associated with mutations in ribosomal protein genes, particularly in L4 and L22, as well as with mutations in the peptidyl transferase region of the 23S rRNA gene (2, 3, 6, 13, 21). The objective of the present work was that of finding macrolide-resistant *C. trachomatis* clinical isolates and studying them for possible mutations in 23S rRNA, L22, and L4 genes.

C. trachomatis isolates were obtained from four patients attending the D.O. Ott Institute of Obstetrics and Gynaecology (Saint Petersburg, Russia) during 2000 to 2002. The reference *C. trachomatis* strain was kindly provided by Eva Hjelm (Uppsala University, Uppsala, Sweden).

McCoy cells were seeded into 24-well cell culture plates (approximately 2×10^5 cells per well) and incubated at 37°C for 24 h to achieve monolayer confluence. For *C. trachomatis* growth in cultures, Eagle's medium containing 0.5% glucose, 10% fetal bovine serum, 25 μg of vancomycin/ml, 25 μg of gentamicin/ml, and 2.5 μg of amphotericin B/ml was used. The cell monolayer was infected with an inoculum which produced 20 to 30 inclusions per field under a magnification of ×400. The infected cells were centrifuged at 1,700 × *g* for an hour and incubated for 2 h at 37°C; after that, the medium was removed and the cells were washed with 0.5 ml of Eagle medium. The infected cells were then overlaid with the growth medium containing serial twofold dilutions of the antibiotic (concentration range, 0.01 to 5.12 μg/ml) and incubated at 37°C for 72 h. The following antibacterial agents were used: erythromycin (Sigma Chemical Co., St. Louis, Mo.) and josamycin and azithromycin (Pfizer/MACK, Illertissen, Germany). The inclusions were detected by direct immunofluorescence using fluorescein-labeled antibodies against major outer-membrane protein (MOMP) (Chlamyset Antigen FA, Orion Diagnostica, Espoo, Finland). The MIC was defined as the lowest concentration at which no typical inclusions were observed. To assay minimal bactericidal concentrations (MBC), the cells were washed from the antibiotic 72 h after infection and the fresh cell monolayer was reinfected. The MBC was defined as that resulting in an absence of inclusions in the monolayer after a 72-hour incubation.

The isolation of a mixture of *C. trachomatis* and McCoy cell genomic DNA and DNA from uninfected McCoy cells was performed by using a standard method of phenol-chloroform extraction. The RNA was isolated using the SV total RNA isolation system (Promega, Madison, Wis.). The nucleotide sequences of the primers used are shown in Table 1. The primers flanking a variable region of the MOMP were used to determine the serotype of each isolate. Sequencing was done using both DNA strands by means of an fmol DNA cycle sequencing system (Promega).

Isolates 1, 2, 4-1, and 4-2 demonstrated resistance to all the macrolides (Table 2). The MIC and MBC of each of the drugs were above 5.12 μg/ml for these isolates (compared to between 0.02 and 0.16 μg/ml for the reference strain). Isolates 3-1 and 3-2 were sensitive to all the drugs. The observed resistance was heterotypic; i.e., the inclusions observed were small and their number in the presence of antibiotics was much lower. Resistant isolates could be visually distinguished from sensitive ones, which showed no inclusions at the drug concentrations defined as the MIC and MBC.

Table 2 shows all the mutations found as a result of sequencing the genes that are coding targets for macrolides. The studied region of gene L4 in all of the isolates showed no difference from that of the published GenBank sequence (NC000117.1). In the L22 gene of all isolates except isolate 1, a triple mutation was found: Gly52(GGC)→Ser(AGC), Arg65(CGT)→Cys (TGT), and Val77(GTC)→Ala(GCC).

Amplification of the peptidyl transferase loop of the 23S rRNA gene was performed with the rr primers. The sequencing of isolates 1, 2, 4-1, and 4-2 with both the forward and reverse primers revealed that at certain positions (the same positions for all isolates), a mixture of termination products was present. At positions 2058 and 2611 (*Escherichia coli* num-

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TABLE 1. Oligonucleotides used for the amplification of fragments of ribosomal protein, MOMP, and 23S rRNA genes

Primer target	Primer and sequence	Position
L4 protein	l4-f, 5′ gaagtttgaattgcctgatgc 3′	45–65[a]
	l4-r, 5′ ggcttaggaccgaaaacaatc 3′	278–258[a]
L22 protein	l22-f, 5′ agctgcaggattgatgagaaa 3′	54–74[a]
	l22-r, 5′ gttagatgactcgtgcgcttc 3′	308–288[a]
23S rRNA	rr-f, 5′ aagttccgacctgcacgaatgg 3′	1952–1973[b]
	rr-r, 5′ tccattccggtcctctcgtac 3′	2675–2656[b]
	rrg-f, 5′ aattccttgtcgggtaagttc 3′	1937–1957[b]
	al1-r, 5′ cgttatgatcccaggatccct 3′	5927–5907[c]
	al2-r, 5′ cccaatatagaaccgaaaattcga 3′	5451–5428[d]
MOMP	Se-f, 5′ ccaatatgctcaatctaacc 3′	1932–1951[a]
	Se-r, 5′ aattgcaaggagacgatttg 3′	2656–2647[a]

[a] Base positions relative to ATG.
[b] *E. coli* numbering.
[c] Position in the sequence at GenBank accession no. AE001345.
[d] Position in the sequence at GenBank accession no. AE001347.

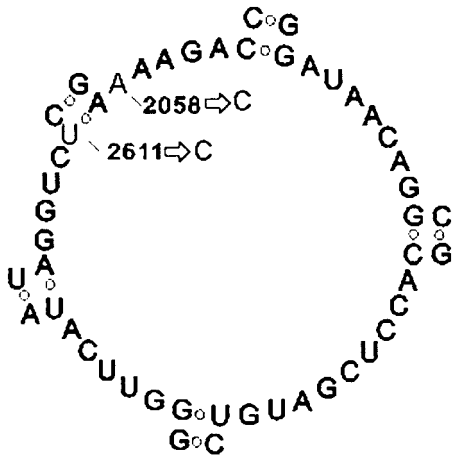

FIG. 1. The secondary structure of the central portion of domain V (the peptidyl transferase loop) of the 23S rRNA gene of a *C. trachomatis* clinical isolate resistant to azithromycin (as determined on the basis of the model by Egebjerg et al.) (2). The arrows show mutations A2058C to T2611C (*E. coli* numbering).

bering)—positions at which mutations are significant for developing drug resistance to macrolides)—both wild-type (A2058 and T2611) and mutant (C2058 and C2611) bases were found. Since the *C. trachomatis* genome contains two copies of the 23S rRNA gene (4), the result described above could mean either the occurrence of mutations in only one copy of the gene in all bacteria or the occurrence of two bacterial populations that were different at those loci.

To determine whether the mutations were homo- or heterozygous, a two-stage amplification of the DNA obtained from isolate 1 was carried out. The first amplification was run with a pair of primers of which the reverse primer was unique for the 3′ region of each operon (al1 and al2) whereas the forward primer was common to both operons (rrg). At the second stage, the product was amplified with the pair of rr primers. The amplicons were then cloned into pGEM-T-Easy vector (Promega). Sequencing of the cloned inserts from six randomly selected plasmids showed that both the wild-type and mutant genes of 23S rRNA had been amplified with each of the reverse primers. Sequencing of the cDNA synthesized from isolated RNAs showed that both variants of the 23S rRNA were produced.

We supposed that we would be able to isolate a resistant population of chlamydiae in the presence of azithromycin if the mutations in the 23S rRNA gene were associated with resistance to macrolides.

We carried out one passage with isolate 1 on cell culture in the presence of 5.12 μg of azithromycin/ml, isolated the DNA, and sequenced it between the two rr primers. At all positions where we had seen a mixture of termination products we now saw only one, corresponding to the mutant version. This fact indicated that mutations in both copies of the 23S rRNA were required for the bacteria to survive in the presence of the drug. Since the peptidyl transferase loops are similar in different microorganisms (21), regions that form the loop in *C. trachomatis* can be identified (Fig. 1). Figure 1 shows mutations A2058C and T2611C. Mutation T2611C destroys the hydrogen

TABLE 2. Results of the assay for antibiotic resistance of *C. trachomatis* clinical isolates and mutations in 23S rRNA and L22 genes

Patient and description	Isolate and date obtained	Antibiotic susceptibility (μg/ml)						Sero-type	Mutation	
		Erythromycin		Azithromycin		Josamycin			23S rRNA[a]	L22[b]
		MIC	MBC	MIC	MBC	MIC	MBC			
1, chronic salpingitis	1, 4/10/2000	>5.12	>5.12	>5.12	>5.12	>5.12	>5.12	G	+	−
2, endocervicitis (the first episode of infection was observed in 1997; spiramycin was used for treatment)	2, 1/5/2000	>5.12	>5.12	>5.12	>5.12	>5.12	>5.12	D	+	+
3, vaginal discharge (treated with josamycin [500 mg] twice daily from 12/12/2001 to 12/21/2001)	3-1 (before treatment), 1/5/2000	0.16	0.16	0.08	0.08	0.04	0.04	B	−	+
	3-2 (after treatment), 10/20/2001	0.16	0.32	0.08	0.08	0.04	0.04	B	−	+
4, urethritis (treated with josamycin [500 mg] twice daily from 6/26/2002 to 7/5/2002)	4-1 (before treatment), 6/17/2002	>5.12	>5.12	>5.12	>5.12	>5.12	>5.12	I	+	+
	4-2 (after treatment), 10/2/2002	>5.12	>5.12	>5.12	>5.12	>5.12	>5.12	I	+	+
C. trachomatis reference strain D/UW-3/Cx (ATCC VR-885)		0.04	0.16	0.02	0.02	0.04	0.08	D	−	−

[a] Double mutations: A2058C and T2611C (*E. coli* numbering).
[b] Triple mutations: Gly52(GGC)→Ser(AGC), Arg65(CGT)→Cys(TGT), and Val77(GTC)→Ala(GCC) (bases relative to ATG).

bond that exists between bases A2057 and T2611. After the single passage in the presence of azithromycin, we performed seven passages of the culture without the drug. After each passage the number of inclusions in the infected monolayer decreased until the isolate died. A similar experiment was also performed with isolate 2, and results were the same.

We therefore concluded that a clinical *C. trachomatis* isolate can contain organisms differing in their levels of susceptibility to antibiotics, some of them resistant to macrolides and carrying mutations in the 23S rRNA gene and others sensitive to macrolides and lacking these mutations. It seems that the viability of bacteria carrying the mutations in both alleles of the 23S rRNA gene is low. The phenomenon of reduced viability of bacteria resulting from acquisition of mutations that lead to drug resistance is well known.

To our knowledge, this is the first time that mutations in the peptidyl transferase region of the 23S rRNA gene have been found in macrolide-resistant *C. trachomatis*. A considerable body of evidence for the interaction of the ribosome with macrolides has been accumulated to date, and it has been demonstrated that the peptidyl transferase region of domain V of the 23S rRNA plays an important role (1, 7, 8, 9, 12, 16, 17, 21). Macrolide-resistant strains of *E. coli* have been isolated that possess a multicopy plasmid expressing a mutant allele of gene rr at position 2058 (18). Subsequently, mutations in this gene have been found in clinical isolates of pathogenic bacteria. In various bacteria the mutation at A2058 is most often associated with a higher level of resistance to all macrolides as well as to lincosamide and streptogramin B, substances that are chemically distinct from macrolides but have a similar mechanism of action in bacterial protein synthesis (11, 15, 18; for a review, see reference 22). Mutations at positions 2057 and 2611 result in disturbance of the hydrogen bond between them and are associated with resistance to erythromycin in propionibacteria (14), *Streptococcus pneumoniae* (20), *E. coli* (5), and *S. pyogenes* (10).

Along with mutations in domain V or II of the 23S rRNA gene, mutations in ribosomal proteins L4 and L22 may also be associated with resistance to various macrolides (2, 13, 20). We have found a triple mutation, Gly52(GGC)→Ser(AGC), Arg65(CGT)→Cys(TGT), and Val77(GTC)→Ala(GCC), in the L22 protein in five out of six studied clinical isolates but have not yet assessed its role in *C. trachomatis* resistance. The mutations reside in a nonconserved region of the L22 protein. Such mutations have not been found in other macrolide-resistant bacteria. Because we have also found these mutations in isolates that were sensitive to macrolides in vitro (3-1 and 3-2), we believe that they are not responsible for macrolide resistance.

In conclusion, it is worth noting that the relationship between the antibiotic resistance manifested by *C. trachomatis* in vitro and the efficiency of antichlamydial therapy has yet to be established. There have been no studies of a representative quantity of *C. trachomatis* clinical isolates characterized by their drug resistance characteristics and compared with respect to the results of patient treatment. The findings for mutations in the 23S rRNA gene associated with *C. trachomatis* resistance to macrolides and described here will facilitate such a study.

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