

Frequency of Development and Associated Physiological Cost of Azithromycin Resistance in *Chlamydia psittaci* 6BC and *C. trachomatis* L2[∇]

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Azithromycin is a major drug used in the treatment and prophylaxis of chlamydial infections. Spontaneous azithromycin-resistant mutants of *Chlamydia psittaci* 6BC were isolated in vitro in the plaque assay at a frequency of about 10^{-8} . Isogenic clonal variants with A₂₀₅₈C, A₂₀₅₉G, or A₂₀₅₉C mutations in the unique 23S rRNA gene (*Escherichia coli* numbering system) displayed MICs for multiple macrolides (i.e., azithromycin, erythromycin, josamycin, and spiramycin) at least 100 times higher than those of the parent strain and were also more resistant to the lincosamide clindamycin. *Chlamydia trachomatis* L2 variants with a Gln-to-Lys substitution in ribosomal protein L4 at position 66 (*E. coli* numbering system), conferring an eightfold decrease in azithromycin and erythromycin sensitivities and a fourfold decrease in josamycin and spiramycin sensitivities, were isolated following serial passage in subinhibitory concentrations of azithromycin. Each mutation was stably maintained in the absence of selection but severely affected chlamydial infectivity, as determined by monitoring the development of each isolate over 46 h in the absence of selection, in pure culture or in 1:1 competition with the isogenic parent. Data in this study support the hypothesis that the mechanisms which confer high-level macrolide resistance in chlamydiae carry a prohibitive physiological cost and may thus limit the emergence of highly resistant clones of these important pathogens in vivo.

The *Chlamydiaceae* are etiological agents of many important human and animal diseases. Formerly called *Chlamydia*, with only two recognized species 25 years ago (*Chlamydia trachomatis* and *Chlamydia psittaci*), the family now contains nine species divided into two genera, *Chlamydia* and *Chlamydophila* (9). As many believe the genus division unnecessary, we refer to both genera as *Chlamydia* for simplicity. These gram-negative bacteria are obligate intracellular organisms that are transmitted as metabolically inactive particles called elementary bodies (EBs). Once inside a host cell, the EB reorganizes morphologically into a reticulate body (RB), within a membrane-bound vacuole known as an inclusion, to grow and replicate by binary fission. Later in the developmental cycle—18 to 48 h postinfection, depending on the species—RBs redifferentiate back to EBs and exit the cell to repeat the cycle (1).

The obligate intracellular nature of these pathogens and their unique biphasic lifestyle pose challenges for the treatment of associated infections (53). Indeed, an efficient anti-chlamydial drug not only must achieve adequate intracellular penetration and concentration but also must be able to inhibit the metabolically active form of the organism (RBs) in its ability to undergo DNA or protein synthesis, cell division, or differentiation into infectious particles (EBs). Currently, the recommended first-line therapeutic regimens for chlamydial infections are the tetracyclines and the macrolide azithromycin (AZM), which inhibit bacterial translation by binding to the

30S and 50S ribosomal subunits, respectively (53). In addition to its chemotherapeutic use, AZM is used for chemoprophylaxis of blinding trachoma, a chronic infection caused by ocular serovars of *C. trachomatis*, common in developing countries (15).

Although chlamydial infections are characterized by a high recurrence rate despite appropriate drug therapy (23), the majority of clinical failures has been attributed to reinfection or relapse following deviation of the organism to persistent, nonreplicating chlamydial forms that are phenotypically antibiotic resistant but can revert to typical RBs at the end of the treatment (22). On the other hand, clinical failures linked to true genotypic resistance due to chromosomal mutations have rarely been reported, suggesting that mutations which confer antibiotic resistance in chlamydiae are not selected for in vivo (23, 61).

Resistance to ribosome-targeting antibiotics due to mutations in rRNA genes has been observed mainly in pathogens possessing a low copy number of rRNA operons, because the selective advantage of a mutation in one rRNA copy is usually masked by the abundance of wild-type drug-sensitive rRNAs transcribed from the other unmutated gene copy (or copies) (49). Interestingly, the *Chlamydiaceae* harbor either one or two rRNA operons depending on the species, implying that resistance to the current anti-chlamydial drugs could emerge from ribosomal mutations. Previously, we showed that spontaneous resistant mutants of *C. psittaci* 6BC with distinct mutations in the unique 16S rRNA gene could be isolated in the plaque assay in the presence of the aminoglycoside spectinomycin (at a frequency of 5×10^{-5}) but not in the presence of tetracycline, another 16S-targeting antibiotic (frequency, $<3 \times 10^{-9}$) (6, 7). Because the physiological burden of resistance muta-

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tions is an important factor that affects the appearance, stability, and maintenance of the phenotype (3, 27), we suggested that chromosomal point mutations conferring resistance to tetracycline incurred a high fitness cost for the bacteria that was reflected in the rarity of genotypic antibiotic resistance among chlamydial clinical isolates. Indeed, stable tetracycline resistance in chlamydiae has been reported only for *Chlamydia suis*, and this resistance probably arose from horizontal gene transfer rather than spontaneous genetic mutations affecting the bacterial ribosome (13).

In this study, we examined the contribution of spontaneous chromosomal mutation to emergence of AZM resistance in chlamydiae in vitro. AZM-resistant chlamydial isolates were isolated either in the plaque assay using a clonal population of *C. psittaci* 6BC (one rRNA operon) or following serial passages in AZM for *C. trachomatis* L2, which harbors two rRNA operons. We found that mutations in the unique *C. psittaci* 23S rRNA gene at position 2058 or 2059 (*Escherichia coli* numbering system) and in *C. trachomatis* ribosomal protein L4 lowered bacterial sensitivities to multiple antibiotics. However, these mutations were associated with a high physiological burden, as evidenced by a reduced production of infectious particles in tissue culture in the absence of antibiotic. The implications of these results for the use of macrolides in treatment of chlamydial infections are discussed.

MATERIALS AND METHODS

Propagation of *C. psittaci*, *C. trachomatis*, and L2 cells. *C. psittaci* serovar 6BC and *C. trachomatis* serovar L2/LGV/434/Bu were grown in mouse fibroblast L2 cells as described in reference 7. Infectious particles from a single plaque purified in the plaque assay from our laboratory stock of *C. psittaci* 6BC were expanded for five developmental cycles to obtain the recent clonal and therefore genetically homogeneous population BC_{RB}. On the other hand, the laboratory stocks of *C. psittaci* 6BC and *C. trachomatis* L2 have been propagated for many years without any kind of clonal purification and are expected to be polymorphic (17).

Titration and antimicrobial susceptibility testing of *C. trachomatis* and *C. psittaci*. The susceptibilities of the chlamydial strains to AZM, erythromycin (ERM), josamycin (JOS), spiramycin (SPI), clindamycin (CLI), virginiamycin M1 (VIR), and chloramphenicol (CLM) were determined in a plaque assay. The MIC was defined as the drug concentration that inhibited the development of 10⁵ chlamydial PFU in a confluent L2 monolayer in a 60-mm dish (7). All antibiotics were purchased from Sigma Chemical Co.

Isolation of chlamydial mutants. To isolate spontaneous AZM-resistant variants, 60-mm dishes were infected with 10⁷ to 10⁸ PFU, corresponding to multiplicities of infection (MOI) of 1 and 10, respectively, and AZM was added 2 h postinoculation (p.i.) at concentrations ranging from 200 ng/ml to 1 µg/ml. The frequency of spontaneous mutation to drug resistance was determined by dividing the number of PFU on selective medium by the number of PFU added to the monolayer (as measured by titration of PFU in the absence of antibiotic) (7).

AZM-resistant populations of *C. trachomatis* L2 were isolated following enrichment by serial passage in subinhibitory concentrations of AZM. Starting with 2 × 10⁹ wild-type *C. trachomatis* L2 infectious particles, we ended up with a population that grew in the presence of 200 ng/ml of AZM after 30 passages.

To monitor the stability of the resistance phenotype, we compared the number of PFU obtained in the absence or presence of antibiotic following the growth of each variant for a minimum of 14 days in the plaque assay in the absence of AZM.

PCR and DNA sequencing of the macrolide resistance targets. PCR amplification and DNA sequencing were used to determine whether resistance to AZM was due to a mutation in the 23S rRNA gene or in *rplD* and *rplV* (encoding L4 protein and ribosomal L22 protein, respectively). PCR primers are listed in Table 1. Total genomic DNA was prepared from infected cells with DNeasy tissue kits (Qiagen). Alternatively, single plaques were collected in 10 µl sterile H₂O and diluted with 230 µl SPG (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid). PCR amplification was then performed on 10 µl of the plaque lysate which had been denatured for 10 min at 96°C beforehand, in a

50-µl reaction mixture using Platinum *Taq* high-fidelity DNA polymerase (Invitrogen) supplemented with 0.8 µg/ml bovine serum albumin. After 30 cycles, the PCR product was ligated in pGEM-T (Promega) and sequenced with primers annealing to the vector (i.e., PUC-R and PUC-F) or to the insert (Table 1).

C. psittaci 6BC *rplD* and *rplV* were amplified with Ultra *Pfu* high-fidelity DNA polymerase (Stratagene) for 15 cycles using primers based on the available genome sequences of the *Chlamydia caviae* guinea pig inclusion conjunctivitis strain, *C. felis* Fe/C-56, and *C. abortus* strain S26/3 (Table 1), cloned in pCRSCRIPT Cam SK(+) (Stratagene) and sequenced on both strands using PUC-F and PUC-R primers.

DNA sequences for each antibiotic resistant isolate were aligned using Clone Manager 8 (Scientific & Educational Software, Durham, NC) and compared to the respective DNA sequence obtained for the wild-type parental strain.

Physiological cost associated with the mutations. (i) Pairwise competition experiment. *C. psittaci* 6BC wild-type BC_{RB} and one isogenic representative of each AZM-resistant variant were coinfecting at a ratio of ~ 1:1 to a MOI of 1 in confluent mouse fibroblast monolayers in 60-mm dishes and incubated at 37°C in 5% CO₂. After 2 h of infection, the inoculum was removed. The cells were washed twice with Dulbecco's modified Eagle medium (DMEM; GIBCO) and incubated in DMEM supplemented with 10% fetal bovine serum, 1× MEM nonessential amino acids (Sigma-Aldrich), and 2 µg of cycloheximide per ml. EBs were harvested in triplicate after sonication of the infected cells at 19, 24, 29, 34, and 46 h p.i. and stored at -80°C in 400 µl SPG. Approximately 8 × 10⁶ infectious particles from the mixed infection obtained at 46 h p.i. were passed a second time into fresh monolayers in 60-mm dishes, allowed to grow for another 46 h, and harvested as before. Titers of serial dilutions of each harvest were determined in duplicate in the plaque assay in both drug-free DMEM (total PFU) and drug-containing DMEM (AZM-resistant PFU). Plaques were counted after 10 days of incubation. The plating efficiency of each resistant mutant was unaffected by the presence of AZM. The twofold PFU increase rate was estimated by using Prism 3.0 software from a plot of ln(PFU) = *f*(time), where the slope is ln2/twofold PFU increase rate (hours). The competition index was defined as the ratio of the output mutant/wild-type ratio to the input mutant/wild-type ratio (6). Additionally, the sizes of a minimum of 60 plaques formed by each *C. psittaci* 6BC strain in the absence of selection at 10 days p.i. were determined and averaged.

(ii) Pure culture. *C. trachomatis* L2 infectivity was determined while the strains were growing in pure culture in the absence of selection. Confluent mouse fibroblast monolayers in 60-mm dishes were infected with each strain to an MOI of 0.1 and incubated at 37°C in 5% CO₂. After 2 h of infection, the inoculum was removed and the cells were incubated in DMEM supplemented with 10% fetal bovine serum, 1× MEM nonessential amino acids, and 2 µg of cycloheximide per ml. EBs were harvested in triplicate after sonication of the infected cells at 46 h p.i. and stored at -80°C in 400 µl SPG. Titers of serial dilutions of each harvest were determined in duplicate in the plaque assay in the absence of selection. Plaques were counted after 14 days of incubation. Sizes of 173 *C. trachomatis* L2 wild-type plaques and 287 and 270 plaques for L₂AZM#23 and L₂AZM#31, respectively, were determined and averaged. The EB generation rate at 46 h p.i. was defined as the number of total PFU obtained at 46 h p.i. divided by the number of PFU used to infect initially.

Nucleotide sequence accession numbers. *C. psittaci* 6BC *rplV* and *rplD* sequences determined in the present study have been deposited in GenBank under accession numbers EU035809 and EU035810, respectively.

RESULTS

Genetic characterization of spontaneous AZM-resistant isolates of *C. psittaci* 6BC. The introduction of single-dose AZM therapy is an important step forward in the treatment of chlamydial infections. AZM, an azalide-macrolide antibiotic, is stable in acidic environments, with additional pharmacokinetic features such as a low plasma concentration, a long half-life of 35 to 40 h, and an elevated penetration in tissues as well as in immune cells. For example, a single 500-mg dose of AZM given to healthy volunteers results in accumulation of the drug to a level of 2 µg/ml in secretions (epithelial lining fluid) (24).

In our laboratory, sensitivity of *Chlamydia* spp. to antibiotics is measured in the plaque assay, and the MIC is defined as the concentration of drug that inhibits the development of 10⁵

TABLE 1. Primers used for PCR amplification and sequencing

Primer target and designation	Position(s) ^a	Sequence (5' → 3')
<i>C. psittaci</i> 6BC 23S rRNA ^b		
6BC8	3415	AGCTGTTGATGGTGACCGTAC
23S-R	4814; C	ACGTATACTAATAGACGCTTAAGAGAG
23SR	4812; C	GTATACTAATAGACGCTTAAGAGAG
<i>C. psittaci</i> 6BC <i>rplD</i> ^c		
L4-F2	108696	CAGAAGAGGTCCTAATGG
L4-R2	109447; C	TTCTCGGTTACATAATGCCG
<i>C. psittaci</i> 6BC <i>rplV</i> ^c		
L22-F2	110879	GGGTAAGTCTAAAGGAGAC
L22-R2	111268; C	TTGGACATCCTTTCTGACCC
DNA insert in pCRSCRIPT Cam or pGEM-T cloning vectors ^d		
PUC-F	847; C	AGCGGATAACAATTTACACAGGAAAC
PUC-R	579	GGTTTTCCCAGTCACGACGTTGT
<i>C. trachomatis</i> L2 23S rRNA ^e		
23S-F1	855946, 877992	TGGTGGATGCCTTGGCATTGAC
AZM-F	857854, 879900	TGAACCTAAGCCCTGGTGAATG
rrn1-R3 (allele 1) ^f	859716; C	TACGTTTGCGGTCCTGCTCTG
rrn2-R (allele 2) ^f	881617; C	CCCTATTCACCCATCGAGAATC
<i>C. trachomatis</i> L2 <i>rplD</i> ^e		
L4-F	595704; C	AAGCGTCTTTCGGGAGTAG
L4-R	594936	GCCTTCTCGGTCACATAATGTC
<i>C. trachomatis</i> L2 <i>rplV</i> ^e		
L22-F	593564; C	GACACAAGTTGGGAGAGTTC
L22-R	593130	GACCACATCCTTACTCTACTG

^a Position in GenBank entry for the first base of the primer. C, complementary strand.

^b Designed according to the sequence of the *C. psittaci* 6BC 23S rRNA (GenBank accession number U68447).

^c Designed based on the respective homologous gene present in the total genome sequences of the closely related *C. caviae* guinea pig inclusion conjunctivitis strain (reference for primer position), *C. felis* Fe/C-56, and *C. abortus* strain S26/3 (GenBank accession numbers AE015925, AP006861, and CR848038, respectively).

^d Designed using GenBank accession number U46018 for the pCRSCRIPT Cam sequence and GenBank accession number X65308 for the pGEM-T sequence. Primer positions in the pCRSCRIPT Cam vector are indicated.

^e Designed based on the *C. trachomatis* D/UW-3/CX complete genome sequence (GenBank accession number AE001273).

^f Allele-specific primers are downstream of the 23S rRNA.

PFU. The number of input bacteria corresponds to an MOI of 0.01 in a confluent monolayer of L2 mouse fibroblasts in 60-mm dishes (7). The AZM MIC is 100 ng/ml for *C. psittaci* 6BC. When monolayers were infected with a minimum of 8×10^7 PFU in the presence of AZM ranging from 200 to 1,000 ng/ml, resistant plaques appeared at a frequency of $1.35 \times 10^{-8} \pm 0.15 \times 10^{-8}$ for the laboratory stock of *C. psittaci* 6BC (population genetically heterogeneous) and $0.75 \times 10^{-8} \pm 0.5 \times 10^{-8}$ for the clonal population BC_{RB}. Fifteen independent AZM-resistant plaques were isolated from both the heterogeneous and the clonal populations of *C. psittaci* 6BC, expanded in the presence of AZM, and further analyzed.

Biochemical studies have shown that AZM reversibly binds to the large ribosomal subunit in the vicinity of the peptidyl transferase center and causes cell growth arrest due to inhibition of protein synthesis (11, 44, 52, 59). More precisely, AZM interacts with bacterial 23S rRNA by connecting hairpin 35 in domain II of the rRNA and the peptidyl transferase loop in domain V. The sequence of the AZM binding site in the 23S rRNA gene was determined by sequencing approximately 900 nucleotides of the 1,400-bp PCR fragment amplified from the parent strain *C. psittaci* 6BC and 30 independent AZM-resistant mutants (Table 2). Each mutant showed a single mutation

at A₂₀₅₈ or A₂₀₅₉ (*E. coli* numbering system), both of which are known to confer the highest levels of macrolide resistance in other organisms (60). One AZM-resistant representative of each mutant class, i.e., BC_{RB}AZ1, BC_{RB}AZ2, and BC_{RB}AZ5, was chosen from the *C. psittaci* 6BC clonal population and expanded for two developmental cycles to perform further phenotypic and physiological characterizations on bacterial populations which were as isogenic as possible (Table 2). Because mutations in ribosomal proteins L4 (*rplD*) and L22 (*rplV*)

TABLE 2. Ribosomal mutations observed in spontaneous AZM-resistant isolates of *C. psittaci* 6BC

No. of independent mutants of <i>C. psittaci</i> 6BC isolated from:		Representative <i>C. psittaci</i> strain ^a	Nucleotide change in the 23S rRNA gene ^b
Laboratory stock	BC _{RB}		
4	7	BC _{RB} AZ2	A ₂₀₅₈ C
4	7	BC _{RB} AZ5	A ₂₀₅₉ C
7	1	BC _{RB} AZ1	A ₂₀₅₉ G

^a Isogenic to the parental isolate BC_{RB}.

^b *E. coli* numbering. No change in the L4 or L22 protein was observed.

TABLE 3. Resistance phenotypes associated with the 23S rRNA alleles in *C. psittaci* 6BC

<i>C. psittaci</i> 6BC strain	23S rRNA mutation ^a	MIC (μg/ml) of:						
		AZM	ERY	JOS	SPI	CLI	VIR	CLM
BC _{RB}	None	0.1	0.2	0.05	1	0.4	2	0.4
BC _{RB} AZ2	A ₂₀₅₈ C	>20	>20	≥10	≥200	40	2	0.4
BC _{RB} AZ5	A ₂₀₅₉ C	>20	>20	>10	>200	5	2	0.4
BC _{RB} AZ1	A ₂₀₅₉ G	>20	>20	>10	>200	40	4	0.4

^a *E. coli* numbering.

have also been reported to confer macrolide resistance in some organisms (12), we amplified, cloned, and sequenced both genes in the isogenic mutant strains. We did not find any changes in the DNA sequences of these two genes. This result indicates that spontaneous AZM resistance arose in *C. psittaci* 6BC from single mutations in the unique 23S rRNA gene, resulting in either an A₂₀₅₈C, A₂₀₅₉C, or A₂₀₅₉G mutation.

Resistance phenotypes associated with the 23S rRNA alleles in *C. psittaci* 6BC. Genetic and biochemical data have shown that macrolides, lincosamides, and streptogramins B bind to the peptidyl transferase center of 50S ribosomal subunits at sites that are close to each other or overlapping (44, 52, 59, 60). Consequently, mutations conferring resistance to a drug belonging to one of these groups of antibiotics may confer cross-resistance to chemically diverse compounds (60). Macrolides are classified according to the size of the lactone ring they harbor (24), and we found that *C. psittaci* 6BC was sensitive to the 14-member-ring ERM, the 15-member-ring azalide AZM, and the 16-member-ring JOS and SPI, with MICs of 200 ng/ml, 100 ng/ml, 50 ng/ml, and 1 μg/ml, respectively, in the plaque assay (Table 3). In addition, we observed growth inhibition of *C. psittaci* 6BC by CLI, a lincosamide antibiotic, and by VIR, a streptogramin A antibiotic, with MICs of 400 ng/ml and 2 μg/ml, respectively, in the plaque assay (Table 3).

Since the three AZM-resistant *C. psittaci* isolates are isogenic, apart from their rRNA mutations, the resistance phenotypes are related directly to the effects of the mutations on drug binding. Although CLM has also been shown to interact with the ribosomal peptidyl transferase cavity (52, 60), BC_{RB}AZ1, BC_{RB}AZ2, and BC_{RB}AZ5 were as sensitive to this drug as the wild-type *C. psittaci* 6BC parent strain BC_{RB}, indicating that none of the mutations in the 23S rRNA at position 2058 or 2059 affected the binding of CLM to the bacterial ribosome (Table 3). On the other hand, A₂₀₅₈C conferred high-level resistance to AZM and ERM and resulted in significant resistance to the 16-membered-ring macrolides JOS and SPI, as well as to the lincosamide CLI, but had no effect on the interactions of VIR with the ribosome (Table 3). This observation has been reported for other organisms (5, 43, 46). As expected, the A₂₀₅₉C and A₂₀₅₉G mutations were associated with high-level resistance to the four macrolides tested but marginally altered the susceptibility of the ribosomes to VIR (Table 3). Interestingly BC_{RB}AZ5, bearing the A₂₀₅₉C mutation in the 23S rRNA, was not as resistant to CLI as the two other variants, harboring an A₂₀₅₈C or an A₂₀₅₉G transition (i.e., 5 versus 40 μg/ml) (Table 3). A similar observation was recently reported for mycobacteria (45).

The stability of the acquired resistance was then tested by

TABLE 4. Drug sensitivity phenotypes associated with the Q₆₆K alteration in *C. trachomatis* L2 ribosomal protein L4

<i>C. trachomatis</i> L2 strain	MIC (μg/ml) of:						
	AZM	ERY	JOS	SPI	CLI	VIR	CLM
Wild type	0.1	0.2	0.05	0.8	0.125	4	0.4
L ₂ AZM#23 ^a	0.8	0.8	0.2	4	0.5	8	0.4
L ₂ AZM#31 ^b	0.8	0.8	0.2	4	0.5	8	0.4

^a Twenty-three passages in various concentrations of AZM (up to 50 ng/ml).

^b Twenty-eight passages with up to 100 ng/ml AZM followed by three expansions without AZM.

growth in the absence of selective pressure (i.e., no AZM). Plaques formed by each *C. psittaci* 6BC variant grown for 14 days in the absence of selection displayed the same number of infectious particles when titers were determined in the presence or absence of 200 ng/ml of AZM, indicating that the resistance phenotype of these three chlamydial mutants was stable (data not shown).

Ribosomal mutations observed in AZM-tolerant isolates of *C. trachomatis* L2. Although *C. trachomatis* L2 has the same sensitivity to AZM as *C. psittaci* 6BC (MIC of 100 ng/ml in the plaque assay), we were unable to obtain spontaneous AZM-resistant isolates of *C. trachomatis* L2 (frequency, $<1.3 \times 10^{-8}$ on 200 and 400 ng/ml AZM and $<4.0 \times 10^{-9}$ on 800 ng/ml and 1.5 μg/ml AZM). Because *C. trachomatis* L2 harbors two copies of the 23S rRNA gene, we hypothesized that a single mutation in only one ribosomal operon might not confer a level of AZM resistance high enough to be selected in one step in the plaque assay. Therefore, we reasoned that serial passage in subinhibitory concentrations of antibiotic would allow the enrichment of putative bacteria with low-level AZM resistance. Starting with 2×10^9 wild-type *C. trachomatis* L2 infectious particles, we isolated an AZM-tolerant population of *C. trachomatis* L2 growing in the presence of twice the MIC after 30 passages with AZM.

Ten individual plaques formed after expansion of the last harvest in the presence or absence of AZM for 2 weeks revealed no mutations in either of the two 23S rRNA genes of the mutants. We also sequenced the *rplD* and *rplV* genes, coding for ribosomal proteins L4 and L22, respectively. These genes represent two other known mutational targets for macrolide resistance (12). In all 10 isolates, one single mutation, C₁₉₆ to A, was found in *rplD*, creating to a Gln-to-Lys alteration at position 66 in the *C. trachomatis* L2 ribosomal protein L4. This mutation arose in the population as early as the 23rd passage (in L₂AZM#23).

Drug sensitivity phenotypes associated with the Q₆₆K alteration in *C. trachomatis* L2 ribosomal protein L4. Gln₆₆ in *C. trachomatis* L2 ribosomal protein L4 corresponds to Gln₆₂ in the homologous *E. coli* protein and lies within the highly conserved region of L4 that is responsible for binding to 23S rRNA (28, 64). The alteration in ribosomal protein L4 conferred an eightfold decrease in sensitivity for AZM, a fourfold decrease in ERM, JOS, SPI, and CLI sensitivity, and a twofold decrease for VIR sensitivity but did not affect the binding of CLM to ribosomes (Table 4). However, the MICs for the macrolides, with the exception of SPI, and the lincosamide were still lower than 1 μg/ml, suggesting that these antibiotics

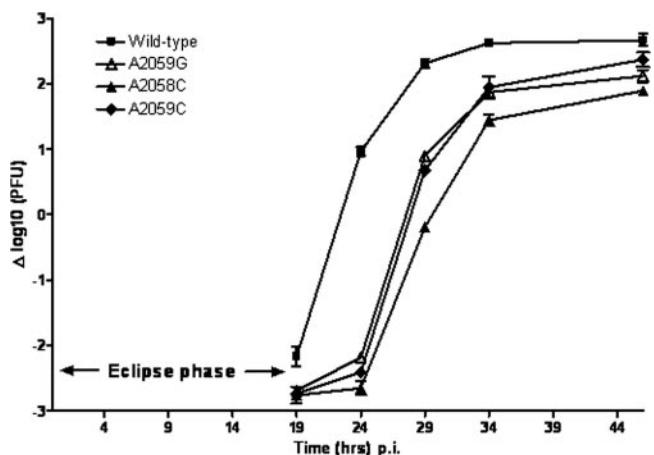


FIG. 1. Growth competition between wild-type *C. psittaci* 6BC and the three isogenic 23S rRNA mutants during one developmental cycle. Infectivity curves for the *C. psittaci* 6BC wild-type strain BC_{RB} and the three isogenic mutants with mutations in the 23S rRNA gene, BC_{RB}AZ1 (A₂₀₅₉G), BC_{RB}AZ2 (A₂₀₅₈C), and BC_{RB}AZ5 (A₂₀₅₉C), were generated by harvesting at 19, 24, 29, 34, and 46 h p.i. Data are means \pm standard deviations of duplicate determinations performed in two separate experiments.

should still show clinical efficacy against these chlamydial mutants in vivo (24, 53).

Physiological costs due to mutations in the 23S rRNA gene associated with AZM resistance in *C. psittaci* 6BC. To be able to survive in a natural environment, a bacterium carrying a mutated allele must compete with the wild-type ancestor population. The outcome of the competition process depends on its relative fitness, defined as the efficiency of multiplication and transmission of the mutant cell compared with that of the wild-type ancestor strain (3). The developmental cycle of *Chlamydia* spp. alternates between replication and growth as RBs and conversion to EBs for transmission (1). In this study, bacterial fitness was assessed by monitoring bacterial infectivity during coinfection of wild-type *C. psittaci* 6BC with each AZM-resistant isogenic variant in L2 cell monolayers in the absence of drug. Figure 1 shows that the eclipse phase, i.e., the period in which infectious progeny EBs have differentiated into non-infectious RBs, lasted longer for the three mutants than for the parental strain, resulting in a severe delay in the RB-to-EB transition. Yet the doubling rate of EB formation during the

TABLE 6. Physiological cost associated with the Q₆₆K alteration in *C. trachomatis* L2 ribosomal protein L4

Strain	Mutation	Plaque size (mm) ^c	EB generation rate (fold increase) ^d
CtL ₂	Wild-type	0.87 \pm 0.24	258 \pm 47
L ₂ AZM#23 ^a	<i>rplD</i> C ₁₉₆ A (K ₆₆ Q)	0.21 \pm 0.12	126 \pm 26
L ₂ AZM#31 ^b	<i>rplD</i> C ₁₉₆ A (K ₆₆ Q)	0.47 \pm 0.18	241 \pm 59

^a Twenty-three passages on various concentrations of AZM (up to 50 ng/ml).

^b Twenty-eight passages with up to 100 ng/ml AZM followed by three expansions without AZM.

^c The size of a minimum of 150 individual plaques was determined for each strain in the absence of antibiotic at 14 days p.i. and averaged.

^d The number of PFU produced at 46 h p.i. divided by the number used at the time of infection.

exponential phase of the cycle was severely affected only for BC_{RB}AZ2, being 1.5 times lower than the rates for the two other mutants and the wild-type strain (44 min versus 29 min) (Table 5) (6). None of the AZM-resistant mutants were able to recover their initial delay in production of infectious particles. Consequently, they were all clearly outcompeted in the absence of selection by the parent strain at the end of the developmental cycle, as reflected by the competition index (CI) (6). With CIs ranging from 0.03 to 0.19 at the end of the first round of competition and 0.006 to 0.09 after the second round (Table 5), it is clear that the AZM resistance mutations A₂₀₅₈C, A₂₀₅₉C, and A₂₀₅₉G in the 23S rRNA gene were associated with a high physiological cost in *C. psittaci* 6BC. The plaques formed by the AZM-resistant *C. psittaci* variants in the absence of selection were 40 to 70% smaller than the parent strain (Table 5). This phenotype also reflects the fitness cost associated with the mutation to resistance.

Physiological costs due to the Q₆₆K mutation in ribosomal protein L4 in *C. trachomatis* L2. The relative decrease in AZM sensitivity incurred by the alteration in *C. trachomatis* ribosomal protein L4 was not high enough to differentiate between the sensitive wild-type strain and the *C. trachomatis* L2 populations harboring the *rplD* C₁₉₆A allele when small inocula were used in the plaque assay (data not shown). Consequently, the bacterial fitness was estimated in pure culture as described in Materials and Methods. We observed a strong biological cost associated with the Q₆₆-to-K change in *C. trachomatis* ribosomal protein L4, illustrated by the formation of smaller plaques and a decrease in the yield of infectious particles at 46 h p.i. (Table 6). Interestingly, additional passages of

TABLE 5. Physiological costs of mutations in the 23S rRNA gene associated with AZM resistance in *C. psittaci* 6BC

<i>C. psittaci</i> 6BC strain	23S rRNA mutation ^a	Plaque size ^b (mm)	Eclipse (h) ^c	2-fold PFU increase rate (h) ^{c,e}	CI ^f at passage:	
					1 (46 h p.i.)	2 (92 h p.i.) ^g
BC _{RB}	None	0.89 \pm 0.31	19	0.48	NA	NA
BC _{RB} AZ2	A ₂₀₅₈ C	0.23 \pm 0.19	24	0.73	0.03 \pm 0.01	0.006 \pm 0.001
BC _{RB} AZ5	A ₂₀₅₉ C	0.54 \pm 0.30	19–24 ^d	0.49	0.18 \pm 0.03	0.07 \pm 0.01
BC _{RB} AZ1	A ₂₀₅₉ G	0.27 \pm 0.10	19–24 ^d	0.49	0.19 \pm 0.03	0.09 \pm 0.01

^a *E. coli* numbering system.

^b The size of a minimum of 50 individual plaques was determined for each strain in the absence of antibiotic at 10 days p.i. and averaged.

^c Data from Fig. 1.

^d Eclipse phase lasted more than 19 h but less than 24 h.

^e Estimated as described in Materials and Methods.

^f Ratio of the output mutant/wild-type ratio at the indicated time to the input mutant/wild-type ratio. NA, not applicable.

^g Determined after a second passage for another 46 h as described in Materials and Methods.

L₂AZM#23 in the presence and absence of AZM allowed the selection of a population that generated as much infectious particles per developmental cycle as the parent strain (Table 6). Nevertheless, L₂AZM#31 still formed plaques that were half the size of those formed by the parent strain, suggesting that this mutant population would still be outcompeted by the parent strain in the absence of antibiotic selection pressure.

DISCUSSION

When cost is not an issue, the macrolide AZM is a major drug used in the treatment as well as in the prophylaxis of chlamydial infections because of the convenient single-dose administration, the high bacterial sensitivity, and the apparent lack of treatment failure due to stable resistance of these obligate intracellular bacteria (23, 53, 61). Additional macrolides, including the 14-member-ring ERM, roxithromycin, and clarithromycin and the 16-member-ring JOS and SPI, also show clinical efficacy against chlamydiae (14, 30, 36, 53). These antibiotics inhibit protein synthesis by reversibly binding to the 50S ribosomal subunit at a site formed by the association of domains II and V of the bacterial 23S rRNA and including the ribosomal proteins L4 and L22, which line the protein exit tunnel (44, 52). Interestingly, lincosamides, such as CLI, and streptogramins, particularly type B components such as pristinamycin IA, inhibit peptide elongation similarly to macrolides, with which they share physically overlapping binding sites on the ribosome (59). Clinical efficacy of CLI has been reported for *C. trachomatis* (2), whereas antichlamydial activity of pristinamycin or virginiamycin has been shown in vitro (26) and in an animal model (35). In this study, we used the plaque assay to confirm the sensitivity of wild-type *C. psittaci* 6BC and *C. trachomatis* L2 to these drugs (Tables 3 and 4) and obtained MICs consistent with those previously reported (25, 55).

Although treatment failures (defined as *Chlamydia* persistence 1 month after treatment) following macrolide therapy have been reported, most reports do not address the role of genetic resistance in the recurrence of chlamydial infections (23, 61). For example, Golden et al. (18) reported a treatment failure rate of 8% among women who reported no new sexual exposures, but that study did not include phenotypic or genotypic susceptibility analysis of the bacteria. Similarly, no genetic analysis was initiated in a patient who failed to clear *C. trachomatis* infection with JOS (51). In veterinary practice, too, Owen et al. reported that infections with *C. felis* (*C. psittaci* feline pneumonitis agent) appeared to be insensitive to AZM in four of five cats studied but did not analyze the possible contribution of mutations to treatment failure (37).

In the present study, high-level AZM resistance arose in *C. psittaci* 6BC at a frequency of around 1×10^{-8} . This frequency is similar to that reported for *Mycobacterium avium*, which, like *C. psittaci*, harbors a single rRNA operon (21, 31). We previously noted a similarity in the development of aminoglycoside resistance by both species (7). Here, we found that spontaneous macrolide resistance arose in *C. psittaci* 6BC from single mutations in the unique 23S rRNA gene resulting in an A₂₀₅₈C, A₂₀₅₉C, or A₂₀₅₉G substitution. This is the first report showing stable, high-level resistance to multiple clinically relevant antibiotics in a chlamydial strain following single point mutations in the 23S rRNA gene. Interestingly, although the

A₂₀₅₈G substitution in 23S rRNA is the most common macrolide resistance mutation encountered in bacterial pathogens (12, 60), it was found in none of the 30 independent mutants of *C. psittaci* 6BC analyzed in this study. This suggests that the A₂₀₅₈G change is deleterious for *C. psittaci* 6BC. Pfister et al. (42) recently proposed that the fitness cost associated with this particular mutation is dependent on the nature of the adjacent 2057-2611 base pair, as bacteria with A₂₀₅₇-U₂₆₁₁, such as mycobacteria, are more tolerant to the resistance mutation than species with G₂₀₅₇-C₂₆₁₁, such as *E. coli*, *Helicobacter pylori*, and *Streptococcus pneumoniae*. However, *C. psittaci* 6BC and other *Chlamydiaceae* display the A₂₀₅₇-U₂₆₁₁ pair as in the mycobacteria. Therefore, one would expect that the biological cost of an A₂₀₅₈G mutation would be tolerated in *C. psittaci* as it is in mycobacteria. We isolated no such mutants. It is possible that, compared to mycobacteria, *C. psittaci* 6BC lacks additional genetic factors such as an intragenic or extragenic compensatory mutation to balance the cost of the A₂₀₅₈G mutation (8, 29). Moreover, no A-to-U transition was observed at position 2058 or 2059 in the AZM-resistant *C. psittaci* mutants, and we did not detect any mutation at positions 2057, 2452, and 2611, although those have been shown to confer low-level macrolide resistance in some other bacteria (60). Because bacteria growing at high density exhibit a low level of antibiotic resistance, we used high concentrations of antibiotic to select for resistant mutants (7). This strategy precluded isolating low-level-resistance isolates of *Chlamydia* by plaque assay. Furthermore, isolation of chlamydial variants by this technique depends on both growth and cell-to-cell transmission of the organism. In the absence of a genetic system for chlamydiae, we cannot determine at this time if any of these mutations would be associated with a level of AZM resistance too low to allow selection in the plaque assay or if the mutations would be deleterious for the organism.

In contrast to *C. psittaci* 6BC, we were unable to obtain highly AZM-resistant isolates of *C. trachomatis* L2 in the plaque assay. We showed previously that the frequencies of resistance to rifampin are on the same order for both chlamydial species (7), suggesting that mutations in the 23S rRNA gene should form at equivalent rates. However, because *C. trachomatis* harbors two 23S rRNA gene copies, selection of spontaneous AZM-resistant isolates in the plaque assay requires the mutation to be dominant over the wild-type (unmutated) copy. Although a resistant phenotype is apparently codominant in mycobacteria (49), *Staphylococcus aureus* (47), *H. pylori* (57), *Mycoplasma hominis* (38), and *Ureaplasma parvum* (40), homogenization of the mutation to all ribosomal copies by gene conversion has been linked to high-level macrolide resistance (32, 56) as well as better stability or homogeneity of the resistance phenotype (4, 57, 62). At the present time, we lack the genetic tools to examine the contribution of single mutations in one 23S rRNA gene copy to the level of macrolide resistance in *C. trachomatis* L2. Interestingly, a double mutation, A₂₀₅₈C T₂₆₁₁C, was recently characterized in clinical isolates of *C. trachomatis* after AZM treatment failure, suggesting that multiple mutations in the 23S rRNA gene are necessary for expression of high-level resistance (30). However, the percentages of heterozygous and homozygous populations are not clear in that report, as both wild-type and mutated copies of the 23S rRNA were detected in the clinical isolates.

In the present study, we isolated a population of *C. trachomatis* L2 with an eightfold decrease in AZM susceptibility due to a mutation in *rplD* coding for ribosomal protein L4. Gln₆₆ (Gln₆₂ in *E. coli*) lies in a phylogenetically conserved disordered loop that displays many basic residues proposed to be the central element in binding of the protein to the 23S rRNA (28, 64). Replacement of the uncharged Gln with the positively charged Lys is likely to affect the binding of the chlamydial ribosomal protein L4 to the cognate 23S rRNA molecules. Indeed, mutations at residues in this conserved L4 disordered loop were found to affect the overall folding of 23S rRNA in domains II, III, and V, perturbing both the translational activity of ribosomes and the action of antibiotics known to interact with nucleotide residues in the peptidyl transferase center (28, 33, 58). Although *rplD* mutations linked to low-level macrolide resistance have been selected in vitro in clinically relevant organisms (10, 39, 54), such mutations are frequently found to be paired with additional mutations in 23S rRNA or in *rplV* (ribosomal protein L22) in clinical isolates (41, 48). Wolter et al. elegantly showed that persistence of macrolide-resistant clinical isolates of *Streptococcus pneumoniae* resulting from alterations in ribosomal protein L4 is linked to acquisition of secondary mutations that compensate for the defect in bacterial growth as well as increasing the level of macrolide resistance (63). In the present work, we found that the physiological cost associated with the Q₆₆K mutation in *C. trachomatis* L4 protein could be alleviated without reversion of the drug sensitivity characteristics of the strain (Table 4 and 6). Further experiments are planned to test whether the new "adapted" genetic background would now be more favorable for acquisition of high-level AZM-resistant mutations.

The rate of increase in frequency of resistance to an antibiotic is directly proportional to the efficacy of the drug and the extent of its use and is inversely proportional to the cost that resistance imposes on bacterial fitness, i.e., its rate of infectious transmission and its ability to compete with other strains (3). We analyzed the biological costs associated with point mutations in the 23S rRNA gene conferring macrolide resistance to *C. psittaci* 6BC by comparing growth of the susceptible parent to that of three isogenic macrolide-resistant variants in the absence of selection. Each mutant was delayed in the formation of infectious particles, as seen by the extended eclipse phase of the development cycle. Additionally, each mutant was severely outcompeted by the wild-type strain at the end of the cycle (CI less than 0.2). These results indicate that mutations in the 23S RNA gene associated with AZM resistance also impose a high physiological cost on *C. psittaci*. It is worth adding that chlamydial growth conditions in this study were optimized for cell culture, i.e., cycloheximide was added to the medium to create a more favorable environment for bacterial growth by inhibiting host protein synthesis, thus making more nutrients available to the bacteria (20). One might expect even stronger developmental differences between each mutant and the parent strain when they have to compete for survival in the natural host. Therefore, we predict that these mutations would not be maintained in vivo unless compensatory mutations are selected to adapt to the costs of chromosomal antibiotic resistance (8, 27, 29).

It is clear that many interacting factors influence the probability of macrolide resistance development in chlamydial in-

fections. Antibiotic-resistant mutants selected in vivo tend to be ones with low or no fitness cost in vitro (16, 34, 50). Unfortunately, the lack of a small animal model for *C. trachomatis* L2 precludes testing the consequence of the change in ribosomal protein L4 on the pathogenicity of the strain. In addition, our laboratory strain of *C. psittaci* 6BC has been passaged in tissue culture for several years, and its current level of virulence in parrots has not been studied. Nevertheless, our results give rise to some general considerations regarding the likelihood of development and persistence of AZM-resistant chlamydial strains. In this study, mutants were readily obtained in vitro for *C. psittaci* 6BC following mutations in the unique 23S rRNA gene, while only *rplD* mutants of *C. trachomatis* L2 with increased tolerance to AZM were isolated. Each mutant strain was attenuated for growth in the absence of selection, suggesting that emergence of AZM resistance in chlamydiae in vivo depends on acquisition of compensatory mutations. Frequent intermittent antimicrobial treatments as a consequence of recurrent infections or repeated mass prophylaxis therapy currently in place to control trachoma in countries where it is endemic (15), as well as for experimental prevention of coronary artery diseases (19), might select for isolates adapted to the high cost of chromosomal resistance. Allowing the *C. trachomatis* *rplD* mutant to evolve during serial passages permitted selection of a more fit bacterial population in vitro without changing the antibiotic sensitivity characteristics of the strain. However, we still lack information on whether chlamydiae are evolving in vivo in response to antibiotic selection pressure. It will be important to examine the consequences of drug resistance mutations on the pathogenicity and survival of chlamydiae such as *C. muridarum* or *C. caviae*, which can be tested in their natural small mammal hosts (i.e., mice and guinea pigs, respectively). Additionally, follow-up of potential treatment failures with both phenotypic and genotypic susceptibility assays of *C. trachomatis* isolates will be important in order to understand whether and how antibiotic resistance occurs in the clinical setting. In the meantime, caution should be used in relying upon AZM for treatment of human and veterinary psittacosis.

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