

Frequency of Spontaneous Mutations That Confer Antibiotic Resistance in *Chlamydia* spp.

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Received 1 December 2004/Returned for modification 30 December 2004/Accepted 18 March 2005

Mutations in rRNA genes (*rrn*) that confer resistance to ribosomal inhibitors are typically recessive or weakly codominant and have been mostly reported for clinical strains of pathogens possessing only one or two *rrn* operons, such as *Helicobacter pylori* and *Mycobacterium* spp. An analysis of the genome sequences of several members of the *Chlamydiaceae* revealed that these obligate intracellular bacteria harbor only one or two sets of rRNA genes. To study the contribution of rRNA mutations to the emergence of drug resistance in the *Chlamydiaceae*, we used the sensitivities of *Chlamydia trachomatis* L2 (two *rrn* operons) and *Chlamydomphila psittaci* 6BC (one *rrn* operon) to the aminoglycoside spectinomycin as a model. Confluent cell monolayers were infected in a plaque assay with about 10^8 wild-type infectious particles and then treated with the antibiotic. After a 2-week incubation time, plaques formed by spontaneous spectinomycin-resistant (Sp^r) mutants appeared with a frequency of 5×10^{-5} for *C. psittaci* 6BC. No Sp^r mutants were isolated for *C. trachomatis* L2, although the frequencies of rifampin resistance were in the same range for both strains (i.e., 10^{-7}). The risk of emergence of *Chlamydia* strains resistant to tetracyclines and macrolides, the ribosomal drugs currently used to treat chlamydial infections, is discussed.

The *Chlamydiaceae*, formerly called *Chlamydia*, are obligate intracellular gram-negative bacteria which were recently divided in phylogenetic studies based on 16S rRNA and 23S rRNA gene sequence analyses into the two genera *Chlamydia* and *Chlamydomphila* (10). In this new taxonomy, *Chlamydia* comprises the species *Chlamydia trachomatis*, its relative *Chlamydia suis*, and *Chlamydia muridarum*, and *Chlamydomphila* currently includes *Chlamydomphila pneumoniae* and *Chlamydomphila pecorum*, in addition to *Chlamydomphila psittaci* and the related *Chlamydomphila felis*, *Chlamydomphila caviae*, and *Chlamydomphila abortus*. *C. trachomatis* and *C. pneumoniae* are mainly pathogens of humans, and the other chlamydial species mainly infect animals (13). However, severe zoonotic diseases have also been reported for *C. psittaci*, *C. abortus*, and *C. felis*. Infections associated with chlamydiae can evolve from a primarily acute phase with relatively mild symptoms to a chronic stage with significant damage to the host from the inflammatory response and tissue fibrosis or scarring. For example, *C. pneumoniae* is a prevalent cause of community-acquired respiratory tract infections and may contribute to chronic pulmonary disease, in addition to Alzheimer's disease and cardiovascular diseases such as atherosclerosis and stroke. *C. trachomatis* is responsible for ocular infections and trachoma that can lead to blindness. *C. trachomatis* is also the major agent of sexually transmitted diseases and can result in pelvic inflammatory disease, infertility, and reactive arthritis (13).

In acute disease, chlamydia development alternates from infectious elementary bodies (EBs) that can survive extracellularly to strictly intracellular reticulate bodies (RBs) that multiply in the cytoplasm of a susceptible host cell within a non-

fusogenic vacuole termed an inclusion (81). At 18 to 48 h postinfection, depending on the species, increasing numbers of RBs convert back to infectious EBs as the remaining RBs continue to multiply until the cell lyses, enabling the EB progeny to pursue the infection by invading neighboring cells. Morphologically aberrant forms of chlamydiae (recently reviewed in reference 31) have been identified during chronic infections (53, 70), as well as in the laboratory in response to adverse environmental conditions such as nutrient depletion (12, 60), inflammatory cytokines (5, 57), and even antibiotic treatment. Indeed, abnormal chlamydial forms have been observed in infected cultures exposed to penicillin (40), ampicillin (79), D-cycloserine (51), sulfonamides (27), fluoroquinolones (16, 24), doxycycline (24), erythromycin (24), and azithromycin (7, 18). The specific inhibition of RB-to-EB differentiation leads to the formation of enlarged, nondividing, but viable chlamydial forms that can potentially revert back to typical RBs with maturation to infectious EBs after removal of the inducer. These so-called persistent bodies are characterized by an abnormal metabolic activity and consequently appear more resistant to conventional antibiotics that target the transcription, translation, or cell division of metabolically active organisms (16, 18, 41, 42).

In recent years, the use and overuse of antimicrobial agents have resulted in an increase in the appearance of antibiotic-resistant bacteria, which poses a growing public health problem. Although chlamydial infections are characterized by a 5 to 20% recurrence rate despite appropriate drug therapy (6, 29, 38, 78), relapses or persistent infections result mainly from patient failure to comply with medication, from repeated infections, or from phenotypic "resistance" associated with the persistent state of the bacteria rather than from real genotypic resistance due to chromosomal mutations (72). Since the chlamydial life cycle occurs in relative isolation, the opportunity for

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TABLE 1. Bacterial strains used for this study

Strain	Description	Source or reference
<i>C. trachomatis</i>		
L2	Biovar lymphogranuloma venereum L2/434/Bu	H. Caldwell
L2R1	Serovar L2 <i>rpoB1</i> , Rif ^r	This work
<i>C. psittaci</i>		
6BC	Serovar 6BC	T. Hatch
BCR1	Serovar 6BC <i>rpoB2</i> , Rif ^r	This work
BC0E1	Serovar 6BC 16S1, Spc ^r	This work
BC0A2	Serovar 6BC 16S2, Spc ^r	This work
BCS18	Serovar 6BC 16S3, Spc ^r	This work
BCS34	Serovar 6BC 16S4, Spc ^r	This work
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 Δ (<i>lacZY-argF</i>) <i>UI169 deoR recA1 endA1 phoA hsdR17 supE44 λ⁻ thi-1 gyrA96 relA1 Δ(<i>lacZ</i>)M15</i>	28

the acquisition of antibiotic resistance genes from other organisms is limited, and indeed, the acquisition of drug resistance in chlamydiae by horizontal gene transfer has been suggested for only one tetracycline-resistant isolate of *C. suis*, a pig pathogen (20). However, studies in the laboratory have shown that *Chlamydia* spp. share the same mutational mechanisms as other bacteria. Experiments involving serial exposures to subinhibitory concentrations of antimicrobials in cell culture have revealed that drug resistance can arise in *Chlamydia* spp. through point mutations that result in the overexpression or alteration of the inhibitor target. For example, increased expression of the ribonucleotide reductase confers resistance to hydroxyurea (67), whereas amino acid substitutions in the CTP synthetase (80), the β subunit of RNA polymerase (RpoB) (17), and DNA gyrase (GyrA) (15) confer stable and high-level resistance in *C. trachomatis* to cyclopentenyl cytosine, rifampin, and the fluoroquinolones ofloxacin and sparflaxacin, respectively.

Nevertheless, we still lack knowledge on the frequency of the appearance of resistance in *Chlamydia* spp. For this study, we determined the frequencies of spontaneous resistance of the sensitive *Chlamydiaceae* to spectinomycin and rifampin by using a plaque assay. Rifampin was chosen due to the highly specific nature of resistance, since single mutations in only one gene, *rpoB*, have been shown to confer high-level resistance on numerous bacteria, including *C. trachomatis* (17). Spectinomycin was used as a model drug for the ribosome-targeted antibiotics currently used to treat chlamydial infections (tetracyclines and macrolides). We show that spontaneous mutations arise in vitro in *C. trachomatis* L2 and *C. psittaci* 6BC at frequencies similar to those in other eubacteria. In particular, the number of ribosomal operons in the organism affected the recovery of spectinomycin-resistant variants but not of rifampin-resistant variants. The implications of these results on the antibiotic treatment of chlamydial infections are discussed.

MATERIALS AND METHODS

Bacterial strains and antibiotics. The bacterial strains used for this study are listed in Table 1. *Escherichia coli* strain DH5 α was used for cloning purposes. *E. coli* was grown in Luria-Bertani broth with aeration or on Luria-Bertani agar. Antibiotics (all purchased from Sigma) were added at the following concentrations, when required: 100 μ g ml⁻¹ ampicillin, 25 μ g ml⁻¹ chloramphenicol, 50 μ g

ml⁻¹ kanamycin, 7.5 μ g ml⁻¹ tetracycline, and 20 μ g ml⁻¹ gentamicin (Gen). Various concentrations of spectinomycin (Spc) and rifampin (Rif) were used.

Propagation of *C. trachomatis*, *C. psittaci*, and L2 cells. *C. trachomatis* serovar L2/LGV/434/Bu and *C. psittaci* serovar 6BC were grown in mouse fibroblast L2 cells. L2 cells were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂. Mouse fibroblast L2 cell monolayers were infected with *Chlamydia* spp. diluted in SPG (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid) at a multiplicity of infection (MOI) of 1. Infected L2 cells were incubated for 44 h in DMEM supplemented with 10% FBS, 20 μ g of Gen per ml, and 1 μ g of cycloheximide per ml. EBs were harvested after sonication of the infected cells, divided into aliquots, and stored at -80°C in SPG.

Titration, antimicrobial susceptibility, and isolation of chlamydial mutants by plaque assay. Confluent monolayers of mouse fibroblast L2 cells in 60-mm² dishes were infected at 37°C in 5% CO₂ with serial dilutions of crude chlamydial stocks. After a 2-h infection time, the inoculum was replaced with an agarose overlay (0.25% Seakem GTG agarose [FMC Bioproducts]) containing DMEM, FBS (10%), cycloheximide (0.2 μ g ml⁻¹), 1 \times NEM nonessential amino acids (Sigma-Aldrich), and Gen (20 μ g ml⁻¹) or various concentrations of Rif or Spc, when appropriate. On day 7, a second agarose overlay containing the same components as the first was added. The cells were stained with 0.5% neutral red for 3 h at 37°C in 5% CO₂ to visualize the chlamydial plaques at the end of the incubation period (between 10 and 14 days postinoculation [p.i.]). Individual plaques were scraped with a pipette tip using 4 μ l of sterile H₂O, suspended in 1.5-ml microcentrifuge tubes that contained 200 μ l of SPG, and frozen at -80°C. Samples were expanded in a plaque assay in the presence of the same concentration of antibiotic used beforehand, and a second round of purification of individual plaques was performed before further characterization.

To monitor the stability of resistance, we expanded each variant for a minimum of 4 days in the absence of antibiotic selection and then collected the variants. The numbers of PFU obtained in a plaque assay in the absence and presence of the antibiotic were compared.

The MIC was defined as the drug concentration that inhibited the development of 10⁵ chlamydial PFU in a confluent L2 monolayer in a 60-mm² dish. This inoculum size resulted in the infection of approximately 1 in 100 cells in the monolayer. For the isolation of spontaneous drug-resistant variants, 60-mm² dishes were infected with 10⁷ to 10⁸ PFU (MOI, 1 to 10), and the drug was added at 2 h p.i. at a concentration high enough to inhibit the cytotoxicity associated with the inoculum size. 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 the titration of PFU in the absence of antibiotics).

Cloning and sequencing of chlamydial *rpoB* and 16S rRNA genes. Total genomic DNAs were prepared from infected cells with DNeasy tissue kits (QIAGEN). To detect changes in the *rpoB* sequence that were associated with rifampin resistance, we amplified the gene with its promoter region by using Ultra Pfu High-Fidelity DNA polymerase (Stratagene) and degenerate primers based on the available genome sequences of *C. trachomatis* serovar D, *C. muridarum*, and *C. caviae* GPIC (GenBank accession numbers NC000117, NC002620, and NC003361, respectively). The 4,294-bp and 4,036-bp fragments amplified from *C. trachomatis* L2 and *C. psittaci* 6BC, respectively, by use of the primers RpoB-F [5'-GGATTAGCTTT(A/G)AAAGAAGCTAAAGAAATGAC-3'] and RpoB-R [5'-CAT(T/C)GTCTCGAGAACCTTCTC(T/C)GAACAT-3'], were cloned into pPCR-Script Cam SK(+) (Stratagene) and sequenced. This experiment was repeated twice to confirm the *rpoB* sequence.

To detect changes in the 16S rRNA gene sequences associated with spectinomycin resistance, we amplified a 1,584-bp DNA fragment encoding the 16S rRNA of *C. psittaci* 6BC by using the primers 16SF1 (5'-AGAATTTGATCTTGGTT CAGATTG-3') and 16SR1 (5'-CCTAGTCAAACCGTCTTAAGACAG-3'), designed from the sequence of the *C. psittaci* 6BC 16S rRNA (GenBank accession number AB001778). The amplified products were cloned into pGEMT (Promega) and sequenced. Alternatively, a 537-bp DNA fragment containing the helix 34 region of the 16S rRNA was amplified by using 16S1 (5'-GCATCTAA TACTATCTTTCTAGAGGG-3') and 16S2 (5'-AACCAGGCAGTCTCGTT AG-3') and then sequenced.

DNA sequences were aligned with Clone Manager 5 software (Scientific & Educational Software, Durham, N.C.) to identify the mutations present in the antibiotic-resistant chlamydial variants.

Southern blot analysis. *C. psittaci* 6BC genomic DNA was prepared by use of a QIAGEN genomic kit from highly purified preparations of EBs obtained by centrifugation through Renocal-76 (Bracco Diagnostics, Princeton, N.J.) density gradients. Aliquots of chromosomal DNA were digested with various restriction endonucleases and analyzed by Southern gel transfer and hybridization with a

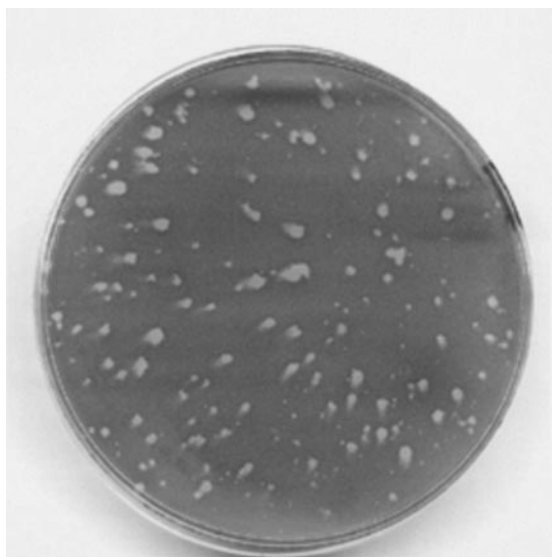


FIG. 1. *C. psittaci* 6BC plaques at 10 days postinoculation. Each plaque, visualized here after neutral red staining of the cells, represents a clonal population of a single infectious particle from the initial infection that can be further purified and expanded.

nonradioactive labeled probe. A 537-bp DNA fragment internal to the *C. psittaci* 6BC 16S gene was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by PCR amplification using the 16S₁ (5'-GCATCTAATACTATCTTTCTAGAGGG-3') and 16S₂ (5'-AACCAGGCAGTCTCGTTAG-3') primers based on the nucleotide sequence of the *C. psittaci* 6BC 16S rRNA gene (GenBank accession number AB001778). Hybridization and immunological detection of the probe were performed as described by Panaud et al. (56), using the enhanced chemiluminescence detection system (Amersham Pharmacia) according to the manufacturer's directions. An analysis of the restriction fragments that hybridized with the probe indicated that *C. psittaci* 6BC contained only one copy of the 16S rRNA gene (data not shown).

Nucleotide sequence accession numbers. The *rhoB* sequences determined in the present study have been deposited in GenBank under accession numbers AY826975 for *C. trachomatis* L2 and AY826976 for *C. psittaci* 6BC.

RESULTS

Use of a plaque assay to measure drug inhibitory concentrations for *Chlamydia* spp. We measured the sensitivities of

C. trachomatis L2 and *C. psittaci* 6BC to rifampin or spectinomycin by using a plaque assay. This assay is based on the ability of these obligate intracellular bacteria to invade and lyse mouse fibroblast L2 cells in a confluent monolayer (3). An agarose overlay containing all of the nutrients necessary to sustain cell viability is applied to the cell monolayer at the end of the invasion time, i.e., at 2 h p.i., so that infectious particles released from the infected cells can only reinfect neighboring cells. Consequently, the initial infection of a single eucaryotic cell by a single EB will lead to the formation of an individual plaque that can be visualized after staining of the monolayer. Plaques formed by *C. trachomatis* were observed at 14 days p.i., as reported previously (48). Plaques formed by *C. psittaci* 6BC were usually observed at 10 days p.i. (Fig. 1).

We observed a dose-dependent effect of rifampin (Rif) and spectinomycin (Spc) on the inhibition of plaque formation for both *C. trachomatis* L2 and *C. psittaci* 6BC (Table 2). We defined the MIC as the lowest concentration of drug that inhibited the visible development of 10⁵ PFU. This inoculum size reflects the standard established for numerous extracellular bacterial strains and corresponds to a multiplicity of infection (MOI) of about 0.01 in a plaque assay using 60-mm² dishes. We observed a slight difference between the Rif MICs for the two chlamydial species, with *C. psittaci* 6BC being more resistant than *C. trachomatis* L2 (MICs of 25 ng/ml and 5 ng/ml, respectively). When the cell monolayer was inoculated with 10⁷ to 10⁸ PFU of either strain in the presence of a minimum of 50 ng/ml of Rif, a few plaques were visualized after staining of the cell monolayer.

The same assay was performed in the presence of spectinomycin (Spc), an aminoglycoside that targets the 30S subunit of bacterial ribosomes. Although *C. trachomatis* L2 and *C. psittaci* 6BC were both sensitive to Spc, with MICs of 40 μg/ml and 20 μg/ml, respectively, only large inocula of *C. trachomatis*, containing up to 5 × 10⁷ PFU, were completely inhibited by the antibiotic at a concentration of 200 or 300 μg/ml. Under the same conditions, a few *C. psittaci* 6BC plaques survived drug selection (Table 2).

Molecular characterization of rifampin and spectinomycin resistance in *Chlamydia* spp. We hypothesized that the plaques surviving a high concentration of antibiotic resulted from in-

TABLE 2. Effect of rifampin and spectinomycin on chlamydial plaque formation

Antibiotic	Drug concn (ng/ml [Rif] or μg/ml [Spc])	<i>C. psittaci</i> 6BC		<i>C. trachomatis</i> L2	
		Input PFU ^a	No. of plaques ^b	Input PFU ^a	No. of plaques ^b
Rif	2	NT	NA	2.4 × 10 ³	Too many to count
	5	NT	NA	2.4 × 10 ⁵	0
	10	4.8 × 10 ⁴	0	2.2 × 10 ⁵	0
	25	4.8 × 10 ⁵	0	2.2 × 10 ⁶	0
	50	2.1 × 10 ⁷	42	2.8 × 10 ⁷	2
	100	10 ⁸	50	2.2 × 10 ⁷	1
Spc	200	10 ⁸	23	6.4 × 10 ⁷	2*
	20	1.1 × 10 ⁵	0	2.4 × 10 ³	Too many to count
	40	2.9 × 10 ⁵	0	2.4 × 10 ⁵	0
	100	2.0 × 10 ⁶	0	1.9 × 10 ⁶	0
	200	2.1 × 10 ⁷	13	2.3 × 10 ⁷	0
	300	10 ⁸	81	4.6 × 10 ⁷	0*

^a The largest inoculum size tested that was not cytotoxic.
^b The average number of plaques (minimum of three independent experiments). NT, not tested; NA, not applicable; *, cell monolayer was unhealthy due to the large concentration of infectious particles.

TABLE 3. Nucleotide differences identified in rifampin- and spectinomycin-resistant isolates of *C. psittaci* 6BC and *C. trachomatis* L2

Mutant strain	No. of independent mutations analyzed		Characteristic of mutant allele ^a				MIC
	Total	Distribution	Designation	Nucleotide change	Representative chlamydial mutant	Allele distribution in drug-resistant organisms	
<i>C. trachomatis</i> L2 Rif ^r	1	1	<i>rpoB1</i>	GAU→GGU (Asp ₅₁₆ →Gly)	L2R1	<i>Mycobacterium tuberculosis</i> , <i>Helicobacter pylori</i>	800 ng/ml
<i>C. psittaci</i> 6BC Rif ^r	1	1	<i>rpoB2</i>	AUG→AUC (Met ₅₁₅ →Ile)	BCR1	<i>M. tuberculosis</i>	300 ng/ml
<i>C. psittaci</i> 6BC Spc ^r	59	52	16S1	C ₁₁₉₂ U	BC0E1	<i>E. coli</i> , <i>Neisseria</i> sp., <i>Nicotiana tabacum</i> chloroplasts	>10 mg/ml ^b
		5	16S2	A ₁₁₉₁ G	BC0A2	<i>Chlamydomonas reinhardtii</i> chloroplasts	>10 mg/ml ^b
		1	16S3	C ₁₁₉₂ G	BCS18	<i>E. coli</i>	>10 mg/ml ^b
		1	16S4	G ₁₁₉₃ C	BCS34	Not previously reported	5 mg/ml

^a Each distinct mutation identified was given an allele name (designation), which represents a specific change (*E. coli* numbering) at the indicated nucleotide in the *rpoB* or 16S rRNA sequence.

^b Spc concentrations above 10 mg/ml were cytotoxic to mouse fibroblast cells in the monolayer.

fection with spontaneous chlamydial variants that were resistant to the drug. Two Rif-resistant (Rif^r) plaques, BCR1 and L2R1, selected from *C. psittaci* 6BC and *C. trachomatis* L2, respectively, in the presence of 200 ng/ml of Rif, and six independent *C. psittaci* plaques selected in the presence of 300 µg/ml of Spc were plaque purified twice and expanded in the presence of the antibiotic. The stability of the resistance acquired was then tested by growth in the absence of selective pressure (i.e., no antibiotic). For the Rif^r variants, a single plaque from L2R1 or BCR1 was first expanded by growth in a 60-mm² L2 cell confluent monolayer for 46 h, harvested, expanded in a 175-cm² flask for another developmental cycle, and then harvested again. For the Spc-resistant (Spc^r) variants, single plaques were purified two successive times in a plaque assay in the absence of the drug. The titers of the final harvests were similar in the plaque assay in the presence or absence of the antibiotic, indicating that the resistance phenotypes of these eight chlamydial mutants were stable (data not shown).

In many bacteria, rifampin resistance is due to mutations in the *rpoB* gene, which encodes the β-subunit of the RNA polymerase (77). We used degenerate primers to amplify the *rpoB* region from L2R1, BCR1, and the two wild-type chlamydial parents and sequenced the genes. The *C. trachomatis* L2 and *C. psittaci* 6BC *rpoB* open reading frames share 79% identity at the nucleotide level and 91% identity at the amino acid level. Single base-pair substitutions were identified in the *rpoB* sequences of the two Rif^r variants, creating two different alleles (designed *rpoB1* and *rpoB2* for L2R1 and BCR1, respectively) (Table 3). Both alleles conferred a high level of Rif resistance on *Chlamydia* spp., with an MIC of 800 ng/ml for *rpoB1* and 300 ng/ml for *rpoB2*. Amino acid substitutions at homologous sites in RpoB have been previously characterized for other Rif^r mutants of bacterial strains (52, 76).

Resistance to Spc due to modifications in the 16S rRNA drug target is well characterized in *E. coli*, *Neisseria* spp., and *Mycobacterium smegmatis* (21; <http://server1.fandm.edu/departments/Biology/Databases/16SMDbexp.html>). Sequencing of the 16S rRNA genes of six independent *C. psittaci* Spc^r isolates indicated that all but one had a C-to-U substitution in the 16S rRNA at position 1192 (*E. coli* numbering) (Table 3). This mutation, designated the *C. psittaci* 6BC mutant 16S1 allele present in the chlamydial isolate BC0E1, confers a high level of Spc

resistance on *E. coli* and *Neisseria* spp. and on *Nicotiana tabacum* chloroplasts. In contrast, 16S2, the allele identified in the BC0A2 Spc^r variant, contains an A-to-G substitution in the 16S rRNA at position 1191 in the *E. coli* numbering system. This mutant allele has been identified only in Spc^r chloroplasts. Both alleles increased the Spc MIC of *C. psittaci* 6BC >500-fold (Table 3).

Frequency of spontaneous mutations conferring rifampin or spectinomycin resistance on *Chlamydia* spp. Each independent antibiotic-resistant variant of *C. trachomatis* L2 and *C. psittaci* 6BC, obtained from individual plaques surviving a high concentration of drug, contained a point mutation in the gene encoding the target of the antibiotic. Consequently, the frequency of spontaneous antibiotic resistance can be determined by dividing the number of mutant plaques that form on selective medium by the number of PFU added to the monolayer. The frequencies of spontaneous Rif resistance were of the same order of magnitude for *C. trachomatis* L2 and *C. psittaci* 6BC, i.e., 2×10^{-7} and 7×10^{-7} , respectively. The rate of spontaneous resistance to Spc for *C. psittaci* 6BC was about 10 times higher, i.e., 10^{-6} (Table 4).

The frequency of mutation measures all mutants present in a given population, irrespective of whether the mutations oc-

TABLE 4. Frequencies of spontaneous mutation to rifampin or spectinomycin resistance in *Chlamydia* spp.^a

Strain	Mutation frequency in presence of drug	
	Rif (200 ng/ml)	Spc (300 µg/ml)
<i>C. trachomatis</i> L2		
Wild type	$1.55 \times 10^{-7} \pm 1.44 \times 10^{-7}$	$<3.60 \times 10^{-8}$
L2R1	NA ^c	$<2.40 \times 10^{-8}$
<i>C. psittaci</i> 6BC		
Wild type	$7.34 \times 10^{-7} \pm 6.23 \times 10^{-7}$	$1.12 \times 10^{-6} \pm 1.70 \times 10^{-6}$
Wild type after three passages ^b	$1.90 \times 10^{-8} \pm 0.7 \times 10^{-8}$	$4.48 \times 10^{-5} \pm 0.89 \times 10^{-5}$
BCR1	NA	$2.23 \times 10^{-7} \pm 1.59 \times 10^{-7}$
BC0E1	$7.54 \times 10^{-8} \pm 3.38 \times 10^{-8}$	NA

^a The frequency of spontaneously resistant mutants was calculated as described in Materials and Methods. Data are means \pm SD.

^b Infectious particles from a single plaque were expanded during three developmental cycles to obtain a recent clonal population of *C. psittaci* 6BC.

^c NA, not applicable.

curred early or late during the growth of the population. Consequently, the Rif and Spc mutation frequencies obtained for our *C. psittaci* 6BC wild-type stock were compared with the values obtained for an early clonal population of the same strain, obtained from a single plaque which was expanded three times. The mutation frequencies were of the same order of magnitude, but with a smaller standard deviation for the recent bacterial population (Table 4). On the other hand, the frequencies of spontaneous resistance obtained with BCR1 and BC0E1 were lower than those seen with the wild-type strain, suggesting some incompatibility between the *rpoB2* or 16S1 allele and additional mutations in the 16S rRNA or the *rpoB* gene, respectively (37). No Spc^r plaques were ever detected with wild-type *C. trachomatis* L2 or L2R1. Therefore, the frequency of spontaneous Spc^r mutation for this species was below the limit of detection, i.e., $< 2.4 \times 10^{-8}$.

Mutations in rRNA genes are generally recessive or codominant, and the expression of the resistant phenotype requires that a majority of the cellular rRNA be of the mutant form (63). Consequently, the number of rRNA operon copies present in the genome affects the frequency of spontaneous resistance to ribosomal antibiotics. Indeed, the genome sequence of *C. trachomatis* revealed the presence of two identical ribosomal operons (73). Our inability to isolate spontaneous Spc^r variants of *C. trachomatis* L2 is consistent with the presence of multiple rRNA operons in this strain. On the other hand, Southern hybridization of a 16S rRNA probe indicated the presence of a single ribosomal operon in the *C. psittaci* 6BC genome (data not shown).

Spectrum of spontaneous Spc^r mutations in *C. psittaci* 6BC. We isolated 53 additional *C. psittaci* Spc^r variants obtained from individual Spc^r plaques purified once and expanded in the presence of the antibiotic. Sequencing of the 16S rRNA helix 34 region revealed that 46 mutants had the same C₁₁₉₂U mutation as that found in BC0E1 and 5 mutants had the same A₁₁₉₁G mutation as that found in BC0A2. Two new alleles conferring high levels of Spc resistance to *C. psittaci* 6BC were identified (Table 3). The 16S3 allele present in BCS18 carried a C₁₁₉₂G mutation that was previously identified in Spc^r *E. coli* (<http://server1.fandm.edu/departments/Biology/Databases/16SMDbexp.html>). Interestingly, the 16S4 allele present in BCS34 harbored a G₁₁₉₃C transition that has never been reported. However, an A substitution at the same position has been identified in Spc^r chloroplasts of *Chlamydomonas reinhardtii*.

DISCUSSION

Because chlamydiae are obligate intracellular bacterial pathogens, drug susceptibilities have traditionally been studied by adding various concentrations of test antibiotics to infected cultured animal cells and detecting the bacteria by direct immunofluorescence. The MIC, the standard measure of antibiotic activity in vitro, has been defined as the lowest antibiotic concentration that completely inhibits the inclusion formation of generally 10³ to 10⁴ infectious particles after 48 to 72 h of incubation in cells grown in 48- or 96-well microtiter plates (25, 75). For our study, susceptibility tests were performed with a plaque assay using a 60-mm² confluent L2 monolayer. This assay uses the ability of the bacteria to lyse the host cells and

form plaques resulting from infection and spreading of a single EB over several successive infection and development cycles. In some aspects, a chlamydial plaque can be seen as similar to a bacterial colony on an agar plate, as each plaque/colony represents a clonal bacterial population that can be purified and expanded. In an early study, Banks et al. showed plaque formation by >100 chlamydial isolates of mammalian, avian, and human origins, including *C. trachomatis* biovar LGV (3). Later, Matsumoto et al. reported plaque formation by *C. trachomatis* serovars C, D, F, H, I, and L2 and by *C. psittaci* Ca110 (47). Recently, the assay was modified to isolate and purify clonal variants of *C. pneumoniae*, including strains AR-39, TW-183, and CWL-029 and the clinical isolate MUL-250 (23). In our study, we extended the plaque assay to *C. psittaci* 6BC and found that it formed plaques with diameters ranging from 0.5 to 1.5 mm at 10 days p.i. When the antibiotic was added at 2 h p.i., we observed that the inhibitory concentration of the antibiotic was dependent on the size of the chlamydial inoculum. This result was expected, as the presence of bacteria growing at a high density can confer a low level of antibiotic resistance (46). Therefore, we defined the MIC as the lowest antibiotic concentration that completely inhibited the plaque formation of 10⁵ infectious particles, as this inoculum size reflects the current standard established for determining MICs for extracellular bacteria (2). Interestingly, the rifampin MIC for *C. trachomatis* in the plaque assay, i.e., 5 ng/ml, was similar to the MIC determined by the traditional methodology, i.e., 7.5 ng/ml, when the same multiplicity of infection (i.e., 0.01) was tested (17). This indicates that the MOI rather than the inoculum size per se is the critical parameter for reproducible MICs for chlamydiae.

Although the *Chlamydiaceae* have been shown to be highly sensitive to rifamycins, the rapid emergence of resistance to these drugs was observed in vitro as early as 1973, and their use in the treatment of chlamydial infections has been avoided (39). Rifampin is a hydrophobic antibiotic and consequently employs passive diffusion to cross the four biological membranes—host cell, inclusion, and RB inner and outer membranes—exerting its inhibitory action by binding to the β subunit of the bacterial RNA polymerase (RpoB). Sequencing of the *rpoB* genes from *C. trachomatis* L2 and *C. psittaci* 6BC revealed that their open reading frames are 91% identical at the amino acid level. Yet *C. psittaci* 6BC appeared five times less sensitive to rifampin than *C. trachomatis* L2. The dissimilarity in sensitivity to rifampin between the two strains may be due to differences in the drug's affinity for the two proteins. It has been shown that serial passaging of *C. trachomatis* in subinhibitory concentrations of rifampin, penicillins, sulfonamides, or fluoroquinolones allows the selection of bacterial populations resistant to the respective antibiotic (72), but the multiple steps involved in this process make it impossible to determine the frequency of drug resistance. Dreses-Werringloer et al. isolated Rif^r *C. trachomatis* variants by using an in vitro model of continuous infection of HEp-2 cells for up to 20 days, with the drug being added at 2 days p.i. (17). A molecular characterization of five Rif^r variants identified two different mutations leading to two amino acid substitutions in RpoB that were previously identified in other Rif^r bacteria. Whereas the chlamydial variants harboring an Ala-to-Val change at codon 522 (*E. coli* numbering) showed a MIC of 4 μg ml⁻¹, the

replacement of His₅₂₆ with Tyr conferred a MIC of either 64 or 256 $\mu\text{g ml}^{-1}$, suggesting that the extended incubation time with the antibiotic selected for multiple mutations conferring a higher resistance level. In addition, the authors demonstrated the presence of extensive genetic variations in each *C. trachomatis* mutant. Consequently, the phenotype observed for these bacterial populations reflected the sum of many individual genotypes.

With the plaque assay, when we infected cells with *C. trachomatis* L2 or *C. psittaci* 6BC at an MOI of >1 , i.e., 10^7 infectious particles, plaques surviving the presence of rifampin at a concentration at least 10 times the MIC appeared for both strains at a frequency of 10^{-7} to 10^{-8} . This is the first time that a frequency of spontaneous mutation has been determined for any phenotype of chlamydiae, and strikingly, this frequency falls within the spontaneous Rif^r frequencies determined for *E. coli* (33), *Helicobacter pylori* (76), *Listeria monocytogenes* (50), and *Mycobacterium tuberculosis* (45). Even though we limited our study to *C. trachomatis* and *C. psittaci*, it is reasonable to suppose that similar types of mutation can arise in other members of the *Chlamydiaceae* because of their close genetic relationship. The RpoB amino acid sequences from two chlamydial Rif^r variants revealed the presence of single substitutions at positions 515 and 516 (*E. coli* numbering) which were previously identified in other Rif^r bacterial species. Whereas Asp₅₁₆ has been recognized as an important Rif binding site, mutations affecting Met₅₁₅ have not been described as frequently for Rif^r bacteria. This amino acid has been identified in close proximity to σ^{70} in the *E. coli* RNA polymerase holoenzyme complex (55). Consequently, mutations at this position may affect the efficacy of transcriptional initiation and could have a serious impact on the physiology of the resistant bacteria.

Tetracycline or its derivative doxycycline and macrolides such as azithromycin and erythromycin are currently the antibiotics of choice for *Chlamydia* spp. These antibiotics target the ribosome by binding to the 16S or 23S rRNA and consequently affect bacterial translation (19). Resistance to ribosome-targeting antibiotics due to mutations in rRNA genes has been observed mainly in pathogens possessing low copy numbers of rRNA operons because the selective advantage of a mutation in one rRNA gene copy is usually masked by the abundance of wild-type drug-sensitive rRNAs transcribed from unmutated gene copies. Interestingly, chlamydiae harbor one or two rRNA operons, depending on the species. Genome sequence analysis or hybridization with an rRNA probe revealed that *C. trachomatis* and *C. muridarum* harbor two homologous rRNA sets, whereas *C. psittaci* 6BC carries a unique rRNA operon, similar to *C. pneumoniae* and *C. caviae* (also called *C. psittaci* GPIC) (36, 61, 62). Therefore, rRNA mutation is a feasible and likely mechanism of resistance to drugs used in clinical settings.

For this study, we used the aminoglycoside spectinomycin (Spc) as a model antibiotic to monitor the potential development of chlamydial resistance by mutations in rRNA genes. Due to their low level of intracellular penetration, aminoglycosides are considered poorly active or inactive against obligate intracellular bacteria, and indeed, their ability to inhibit chlamydial growth is essentially nil at any clinically relevant concentration. However, earlier reports indicated that high concentrations of Spc could inhibit the development of *C.*

trachomatis and *C. psittaci* in vitro (34, 43). We confirmed these observations with our plaque assay and showed that the Spc MICs for *C. trachomatis* L2 and *C. psittaci* 6BC were 40 and 20 $\mu\text{g/ml}$, respectively. In addition, we clearly demonstrated that distinct mutations within the 16S rRNA gene result in Spc resistance in *C. psittaci* 6BC. The 59 independent mutants analyzed carried a total of four different single point mutations located in the upper stem of the phylogenetically conserved helix 34. Data obtained from crystal structure, chemical footprinting, and mutagenesis experiments revealed that Spc interacts directly with the G₁₀₆₄-C₁₁₉₂ base pair (*E. coli* numbering system), where it inhibits peptidyl-tRNA translocation by preventing the binding of elongation factor G to the ribosome (8, 11). As expected, the majority of *C. psittaci* 6BC Spc^r isolates had a mutation at position 1192, therefore perturbing the drug-binding pocket. The rest of the mutations affected the neighboring nucleotides at positions 1191 and 1193, which were previously characterized only for Spc^r chloroplasts. Interestingly, phylogenetic and genomic analyses have linked the *Chlamydiaceae* with the cyanobacterial/chloroplast lineage (9). However, expression of the A₁₁₉₁G mutation in recombinant *E. coli* strains gave rise to Spc^r, suggesting that the low content of rRNA operons shared by *C. psittaci* and tobacco or *Chlamydomonas* chloroplasts, rather than their proposed phylogenetic relationship, was involved in the recovery of such mutants (R. Binet and A. T. Maurelli, unpublished results). Whereas additional mutations at positions 1066 and 1064 have been associated with Spc^r in *E. coli*, chloroplasts, and *Neisseria meningitidis*, we have not found any evidence of Spc^r-associated mutations at equivalent locations within the 16S rRNA gene of any resistant *C. psittaci* strain. In the absence of a genetic system for chlamydiae, we cannot determine at this time if any of these mutations would be deleterious to the organism.

C. psittaci 6BC Spc^r variants arose at a frequency of 5×10^{-5} . On the other hand, the development of Spc resistance in *C. trachomatis* L2 was below the level of detection of the plaque assay, i.e., $<2.4 \times 10^{-8}$, in agreement with the larger ribosomal operon copy number in this strain. In support of our results, the frequency of appearance of spontaneous resistance to the aminoglycoside amikacin is as high as 10^{-5} for *Mycobacterium chelonae* and *Mycobacterium abscessus*, which contain a unique ribosomal operon (59). On the other hand, *M. smegmatis* fails to develop spontaneous mutants resistant to the aminoglycoside hygromycin B unless one of the two chromosomal rRNA copies is previously inactivated (58). Thus, it appears that the expression of aminoglycoside resistance requires that $>50\%$ of the ribosomal population be of the resistant phenotype (63). On the other hand, tetracycline or macrolide resistance occurs when at least 50% of the ribosomal population is mutated (4, 14, 22, 32, 69). Interestingly, these differences reflect the respective growth inhibitory activities of these different families of antibiotics, as aminoglycosides are generally bactericidal, whereas macrolides and tetracyclines are mostly bacteriostatic (74). In clinical strains, however, rRNA genes are generally homozygous, likely following gene conversion, a form of recombination resulting in the conversion of one allele to another (30). The homogenization of the genotype seems to be linked to more stability or homogeneity of the resistance phenotype. For example, the heterogeneous

expression of erythromycin resistance in clinical isolates of *Bordetella pertussis*, of which both sensitive and resistant organisms were cultured in vitro, results from heterogeneity in the number of mutated 23S rRNA gene copies relative to the number of wild-type copies present in the organisms (4). Consequently, the "heterotypic" phenotypes described for *C. trachomatis* clinical isolates that are resistant to tetracycline (35, 44, 71) or azithromycin (49) could also result from a heterozygous genotype. Interestingly, Misyurina et al. detected both wild-type and mutated copies of 23S rRNA in azithromycin-resistant clinical isolates of *C. trachomatis*, although it was not clear what percentage of the population was heterozygous or homozygous (49). Additional passages in the presence of azithromycin allowed homogenization of the genotype but resulted in a loss of bacterial viability, suggesting that these resistant clinical isolates would not survive in nature. This apparent decrease in *C. trachomatis* fitness associated with the 23S rRNA macrolide resistance mutation could explain why, although treatment failures have also been reported for the use of macrolides for *C. pneumoniae* (26, 29, 65, 66) and *C. felis*, which are closely related to *C. psittaci* (54), no genetic evidence of macrolide resistance was detected in the few isolates retrieved (64). In addition, attempts to isolate macrolide-resistant variants of *C. pneumoniae* in vitro by repeated passaging in the presence of clarithromycin or azithromycin have failed (64, 68), even though a *C. pneumoniae* quinolone-resistant variant resulting from a point mutation in the gyrase gene was isolated recently in vitro by the same procedure (68).

In an era in which antibiotic resistance is a growing public health concern, knowledge regarding the frequency as well as the biological consequences of resistance may be our best strategy to counter it. We showed here that spontaneous mutations arise in vitro in *C. trachomatis* L2 and *C. psittaci* 6BC at frequencies similar to those for other eubacteria. In particular, the frequencies of spectinomycin resistance in *C. psittaci* 6BC and *C. trachomatis* L2 were comparable to those established for mycobacteria with regard to the number of rRNA copies present in the bacterial strains. In addition, because resistance to macrolides or tetracyclines is dominant in a merodiploid strain, resistance to the drugs currently used to treat chlamydial infections could emerge from single mutations whether the strain harbors one rRNA copy, such as *C. psittaci* and *C. pneumoniae* strains, or two rRNA copies, such as *C. trachomatis* strains, provided that the mutation does not confer a physiological burden. Indeed, whether drug-resistant organisms persist in nature depends on their likely occurrence as well as their fitness relative to their drug-sensitive counterparts (1). We are currently investigating the fitness costs due to mutations in the 16S rRNA associated with spectinomycin resistance in *C. psittaci* 6BC. This will provide additional information on the use of the plaque assay as an effective method to predict, monitor, and perhaps prevent the emergence of antibiotic resistance in chlamydiae.

ACKNOWLEDGMENTS

This work was supported by grant AI44033 from the National Institute of Allergy and Infectious Diseases.

We thank H. Caldwell for the gift of *C. trachomatis* serovar L2, T. Hatch for the gift of *C. psittaci* serovar 6BC, and M. N. Flora and the USUHS Biomedical Instrumentation Center for DNA sequencing and

oligonucleotide synthesis services. R.B. also gratefully acknowledges S. Létoffé and A. J. McCoy for their stimulating comments.

The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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