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Impact of Azithromycin Resistance Mutations on the Virulence and Fitness of *Chlamydia caviae* in Guinea Pigs[▽]

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Azithromycin (AZM) is a major drug used in the treatment and prophylaxis of infections caused by *Chlamydia*, yet no significant clinical resistance has been reported for these obligate intracellular bacteria. Nevertheless, spontaneous AZM resistance (Azm^r) arose *in vitro* at frequencies ranging from 3×10^{-8} to 8×10^{-10} for clonal isolates of *Chlamydia caviae*, which is a natural pathogen of guinea pigs. Sequencing of the unique 23S rRNA gene copy in 44 independent Azm^r isolates identified single mutations at position A_{2058} or A_{2059} (*Escherichia coli* numbering system). While SP_6AZ_1 ($A_{2058}C$) and SP_6AZ_2 ($A_{2059}C$) Azm^r mutants showed growth defects in cell culture and were less pathogenic in the guinea pig ocular infection model than in the parent SP_6, the three isogenic *C. caviae* isolates grew equally well in the animal. On the other hand, coinoculation of the *C. caviae* parent strain with one of the Azm^r strains was detrimental for the mutant strain. This apparent lack of association between pathology and bacterial load *in vivo* showed that virulence of the two Azm^r mutants of *C. caviae* was attenuated. While chlamydial growth *in vitro* reflects the ability of the bacteria to multiply in permissive cells, survival in the host is a balance between cellular multiplication and clearance by the host immune system. The obligate intracellular nature of *Chlamydia* may therefore limit emergence of resistance *in vivo* due to the strength of the immune response induced by the wild-type antibiotic-sensitive bacteria at the time of antibiotic treatment.

Chlamydiae are Gram-negative obligate intracellular pathogens that are responsible for a multitude of diseases in humans and in animals. They employ a biphasic developmental cycle consisting of environmentally stable and metabolically inactive but infectious elementary bodies (EBs), responsible for transmission, and strictly intracellular, metabolically active, and replicative reticulate bodies (RBs) (1). Infection starts when one EB is endocytosed by a susceptible eukaryotic cell and resides within a cytoplasmic vacuole termed the inclusion, where it transforms into the RB. After replicating by binary fission, RBs transform back to the infectious form at 18 to 48 h postinfection, depending on the species, before being released to the cell's exterior, where they start a new round of infection in neighboring host cells. Although chlamydiae may cause direct damage to the epithelium through this cycle of infection and cell lysis, the primary pathology is mediated by the host response to the infection (28, 33). Chlamydial infections are indeed characterized by an initial intense inflammation, which is a major cause of the pathology, while repeated or chronic infections may result in increased tissue damage and scarring via acute inflammation and a T-cell-mediated response.

The currently recommended first-line therapeutic regimens for chlamydial infections are 1 week of doxycycline, a tetracycline (Tet) derivative, or a single dose of the macrolide azithromycin (AZM) (31). AZM is also used for chemoprophylaxis of blinding trachoma, a chronic infection caused by ocular serovars of *Chlamydia trachomatis*, common in underdeveloped and developing countries (12). Tet and macrolide antibiotics inhibit bacterial translation by binding to the 30S and 50S ribosomal subunits, respectively. Although extensive use of drugs has been known to favor the selection of resistance in just about every other bacterial pathogen, including *Chlamydia suis* in the pig (11), treatment failures for *Chlamydia* have been attributed mostly to reinfection rather than real genotypic resistance due to chromosomal mutations (15, 36). However, we showed previously that the low number of rRNA operons in *Chlamydia* presents an actual risk for emergence of resistance against the current preferred therapies. Accordingly, we have shown that the frequency of spontaneous resistance to spectinomycin (5), kasugamycin (7; R. Binet and A. T. Maurelli, submitted for publication), or AZM and other macrolides (6) is at least 2 to 3 logs higher in *Chlamydia psittaci* 6BC harboring a single rRNA operon than in *C. trachomatis* serovar L2 with two rRNA chromosomal copies. Only a low level of AZM resistance (0.8 μg/ml) was obtained for *C. trachomatis* L2 in the laboratory because of a Gln-to-Lys substitution in ribosomal protein L4, which is encoded by a single gene in the bacterial chromosome (6). Because the physiological burden of resistance mutations is an important factor that affects the appearance, stability, and maintenance of the phenotype (2, 19), we suggested that the continued good clinical efficacy of AZM for *Chlamydia* is linked to the deleterious effects conferred by antibiotic resistance mutations on bacterial fitness, including growth rate, transmission, or virulence. Each antibiotic-resistant mutant of *C. trachomatis* L2 or *C. psittaci* 6BC tested is generally affected in its ability to grow and compete with the

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isogenic parent strain in cell culture. Thus, we can assume that these chlamydial variants would not be as pathogenic to humans as the parent strain unless secondary mutations can compensate for the fitness cost originally incurred by the antibiotic resistance mutation (6).

Studying host-pathogen interactions in humans presents limitations that can be overcome with the use of animal models. For example, guinea pigs infected intravaginally with *C. caviae*, the agent of guinea pig inclusion conjunctivitis (GPIC), develop a disease which remarkably parallels the human chlamydial genital infection with regard to pathogenesis, pathology, and immunology (26). In addition, ocular infection of guinea pigs with *C. caviae* generates an acute conjunctivitis that is easily monitored. Hence, this study aimed at analyzing the biological costs of point mutations in the 23S rRNA gene conferring Azm^r to *C. caviae*, in cell culture as well as *in vivo* in its natural host. Although changes from A to C at position 2058 or 2059 in the 23S rRNA gene lowered the production of infectious particles in cell culture by *C. caviae*, we did not notice any differences between the abilities of Azm^r mutants and the wild-type strain to survive in the host in the absence of antibiotic selection. The *in vivo* fitness cost conferred by the resistance mutations was seen only in the intensity of the pathological response, and after experiments measuring competition between each Azm^r mutant isolate and its isogenic parent in the host. The implications of these results on the biology and potential for emergence of antibiotic resistance in *Chlamydia* are discussed.

MATERIALS AND METHODS

Titration, antimicrobial susceptibility testing, and isolation of *C. caviae* mutants in the plaque assay. The susceptibilities of *C. caviae* strains to AZM, purchased from Sigma Chemical Co., were determined in a plaque assay using mouse fibroblast L2 cells as described in reference 5. The MIC was defined as the drug concentration that inhibits the development of 10^5 chlamydial PFU in a confluent L2 monolayer in a 60-mm dish. A maximum concentration of 20 μg/ml AZM was tested, as this concentration was already 10 times the physiological level attained in the body during treatment (16). To isolate spontaneous Azm^r variants, confluent monolayers grown in 60-mm dishes were infected with 10^7 to 10^8 PFU, corresponding to multiplicities of infection (MOIs) of 1 and 10, respectively, and AZM was added at 2 h postinoculation (p.i.) at concentrations ranging from 0.5 to 2 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) (5).

To monitor the stability of the resistance phenotype in *C. caviae* SP_6AZ_1 and SP_6AZ_2, we compared the numbers of PFU obtained in the absence and presence of antibiotic following the growth of each variant for a minimum of 14 days in the plaque assay in the absence of AZM. Sizes of a minimum of 100 plaques were determined at 7 days p.i. and averaged.

PCR and DNA sequencing of the macrolide resistance targets. Total genomic DNA was prepared from infected cells with DNeasy tissue kits (Qiagen). Part of the *C. caviae* 23S rRNA gene was amplified by PCR using primer RB45 (5′-CACACCCAACCTATCAAC-3′) and primer 6BC8 (5′-AGCTGTTGATGGTGACCGTAC-3′), purified, and then sequenced using primer AZM-F (5′-TGAACCTAAGCCCTGGTGAATG-3′) as described previously (6). DNA sequences for each antibiotic-resistant isolate were aligned using Clone Manager 9 (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) Pure culture. *C. caviae* infectivity was determined while the strains were growing in pure culture in the absence of antibiotic selection. Confluent mouse fibroblast monolayers in 60-mm dishes were infected with 3×10^5 inclusion-forming units (IFU) of *C. caviae* wild-type SP_6, 1×10^6 IFU of SP_6AZ_1, or 9×10^5 IFU of SP_6AZ_2 and incubated at 37°C in 5% CO_2. After 2 h of infection, the inoculum was removed and the cells were incubated in infection medium, i.e., Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× MEM nonessential amino acid solution (Sigma-Aldrich), and 2 μg of cycloheximide per ml. Duplicate dishes were harvested at various times after inoculation. The supernatants were removed, centrifuged at 13,000 rpm for 10 min, resuspended in 400 μl of SPG (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid), pulse-sonicated, and stored at −80°C until they were thawed for determination of the progeny IFU released in the growth medium. Infected cells were scraped from each dish with a rubber scraper, rinsed with 400 μl of SPG, collected into a tube, pulse-sonicated, and stored at −80°C until they were thawed for determination of the progeny IFU contained in the infected cells.

For quantification of *C. caviae* infectious titers, supernatant and pellet harvests were serially diluted in infection medium and 0.2 ml of each dilution was centrifuged (3,000 rpm, 1 h, 37°C) onto duplicate cultures of McCoy cells grown in flat-bottom 96-well plates (Costar). Plates were incubated for 1 h at 37°C before replacement of the inocula with 0.2 ml of infection medium supplemented with 0.2 μg/ml AZM, when appropriate. After 24 h of incubation, cells were fixed with methanol and stained with a fluorescein isothiocyanate (FITC)-conjugated *Chlamydia* genus-specific monoclonal antibody (MAb) (Pathfinder *Chlamydia* culture confirmation system; Bio-Rad). Chlamydial inclusions were counted by fluorescence microscopy and the results expressed as numbers of IFU per sample. For clarity, the number of IFU in the pellet was added to the number of IFU in the supernatant to represent the total number of EBs in the wells. In the infectivity curves shown in Fig. 1, the numbers of EBs produced at 19, 22, 25.5, 29, 35, and 43 h p.i. were related to the number of infectious particles at the time of infection. The rate of 2-fold EB increase was estimated using the Prism 3.0 software program from a plot of $\ln(\text{PFU}) = f(\text{time})$ (with goodness-of-fit [r^2] values of 0.98 for SP_6 and 0.99 for SP_6AZ_1 and SP_6AZ_2), where the slope is ln2/rate of 2-fold EB increase (h). The rate of EB production is the number of IFU produced at 43 h p.i. divided by the number of infectious particles initially used for infection.

(ii) Pairwise competition experiment. Wild-type *C. caviae* SP_6 and one isogenic representative for each Azm^r variant were coinfected at a ratio of ~1:1 to an MOI of 0.1 in confluent mouse fibroblast monolayers in 60-mm dishes and incubated at 37°C in 5% CO_2. After 2 h of infection, the inoculum was removed. The cells were washed twice with DMEM and incubated in infection medium. EBs were harvested in triplicate after sonication of the infected cells at 43 h p.i. and stored at −80°C in 400 μl SPG. Approximately 5×10^5 infectious particles from the mixed infection obtained at 43 h p.i. were passed a second time into fresh monolayers in 60-mm dishes, allowed to grow for another 43 h, and harvested as before. The titers of serial dilutions of each harvest were determined in duplicate using McCoy cells grown in 96-well plates, as described above, in both drug-free (total IFU) and drug-containing (Azm^r IFU) DMEM. The competition index (CI) was defined as the ratio of the output mutant/wild-type ratio to the input mutant/wild-type ratio (4).

Guinea pigs. Female Hartley strain guinea pigs, each weighing 450 to 500 g, were obtained from Charles River Laboratories (Boston, MA). All animals were housed individually in cages covered with fiberglass filter tops and given food and water *ad libitum* and maintained on a 12:12 light-dark cycle. Each experimental group routinely consisted of five animals. All animal experiments and protocols were approved by the Animal Care and Use Committee of the Arkansas Children's Hospital Research Institute.

Conjunctival infection of guinea pigs. Guinea pigs were infected in the conjunctiva of both eyes by instilling 20 μl of SPG containing 10^4 IFU of *C. caviae* directly into the conjunctival sac. This dose ensures 100% infection and produces a strong pathological response that is easily quantified by gross observation. Pathological changes were assessed daily on each eye by using a 0-to-4+ scale while evaluating palpebral and bulbar conjunctiva for erythema, edema, and exudation (27). The scores are defined as follows: 1+, slight erythema or edema of either the palpebral or the bulbar conjunctiva; 2+, definite erythema or edema of either the palpebral or the bulbar conjunctiva; 3+, definite erythema or edema of both the palpebral and the bulbar conjunctiva; 4+, definite erythema or edema of both the palpebral and the bulbar conjunctiva and the presence of exudate. In order to maintain consistency, only one individual evaluated the pathology. Conjunctival material for the isolation and quantification of chlamydiae was collected from the conjunctiva by using a Dacron swab and then placing the swab in sucrose-phosphate transport medium. There was no effect of the swab collection on the conjunctival pathology. Numbers of IFU were determined by culture in McCoy cells. In order to quantify the AZM mutants, swab material was cultured in the presence of AZM. The total number of wild-type IFU was then determined by subtracting the number of IFU obtained in the AZM culture from that obtained in the culture without AZM. Groups of animals were compared statistically using a 2-factor (group and time) analysis of variance (ANOVA) with repeated measures.

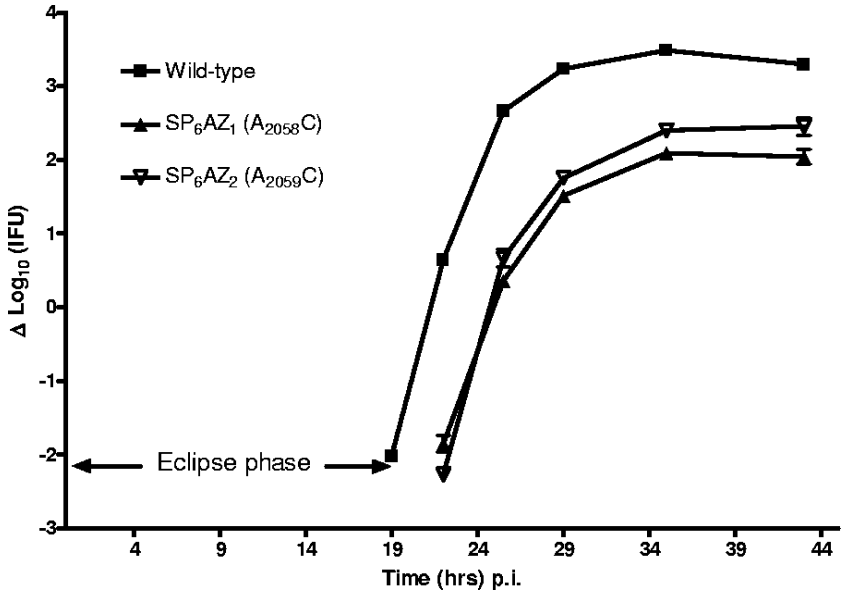

FIG. 1. Impact of AZM resistance mutations on the growth of *C. caviae* SP_6 variants in mouse fibroblast L2 cells. Confluent mouse fibroblasts in 60-mm dishes were infected with wild-type *C. caviae* SP_6 and two isogenic mutants with mutations in the 23S rRNA gene, SP_6AZ_1 ($A_{2058}C$) and SP_6AZ_2 ($A_{2059}C$). The growth medium from each dish (i.e., culture supernatant) and then the infected monolayers were collected at regular time intervals. Infectious particles were harvested into 400 μl SPG. IFU were enumerated after infection onto McCoy cells and immunodetection of the chlamydial inclusions at 24 h p.i. The total number of infectious particles was determined by adding both titers, i.e., those of the supernatant and the infected monolayer, at the respective time point. Data are expressed as means ± standard deviations of results from triplicate determinations and are related to the number of IFU added to the monolayer.

RESULTS

Isolation of distinct clonal populations of virulent *C. caviae*. Guinea pig inclusion conjunctivitis is a naturally occurring chlamydial disease which is normally self-limiting and leaves the animal partially resistant to reinfection. The causative agent was first recovered from conjunctival scrapings by Murray in 1964 (22) and was obtained by Almen Barron at passage 5 or 6 in the late 1960s. It has been subsequently propagated in the Barron/Rank laboratory since that time, first in chick embryos and then in tissue culture, without any kind of clonal purification. Consequently, *C. caviae* laboratory stocks are expected to be very polymorphic. Clonal virulent populations of *C. caviae* were prepared by purifying and then expanding single plaques formed by the bacteria collected from the eye of an infected guinea pig in the plaque assay. This assay is based on the ability of the bacteria to invade into, replicate within, and lyse mouse fibroblast L2 cells in a confluent monolayer (3). An agarose overlay containing all the nutrients necessary to sustain cell viability is applied to the cell monolayer at the end of the invasion period so that infectious particles (EBs) released from the infected cells can reinfect neighboring cells only. Therefore, the initial infection of a single eukaryotic cell by a single EB will lead to the formation of an individual plaque that can be visualized by staining the monolayer, usually after 5 to 7 days for *C. caviae*; purified; and serially expanded to obtain a clonal bacterial population. Accordingly, we prepared 10 clonal isolates of *C. caviae* (SP_1 to SP_{10}) originating from the eye of a guinea pig infected 9 or 12 days earlier (Table 1).

Genetic characterization of spontaneous Azm^r variants from virulent clonal *C. caviae* isolates. To select for resistant mutants, we generally infect confluent monolayers in 60-mm dishes with 10^7 to 10^8 infectious particles at concentrations of antibiotic high enough to inhibit the large inoculum (5–7). Accordingly, the 10 clonal populations of *C. caviae* obtained after four serial expansions of 10 individual plaques in the absence of drug were grown in the plaque assay in the presence of 0.5 to 2 μg/ml AZM. Plaques surviving the antibiotic pressure appeared at frequencies ranging from about 3×10^{-8} to 8×10^{-10}, depending on the isolates, highlighting the variability and possibly genetic differences between the different clonal isolates of *C. caviae* (Table 1). Forty-five spontaneous Azm^r plaques were isolated and expanded in the presence of AZM for DNA analysis as previously described. Each resistant mutant showed a single mutation in the unique 23S rRNA gene at position A_{2058} or A_{2059} (*Escherichia coli* numbering system); both of these mutations are known to confer the highest levels of macrolide resistance in other organisms (35), including *C. psittaci* 6BC (6). Two independent Azm^r mutants (i.e., SP_6AZ_1 and SP_6AZ_2) (Table 1) were then compared for growth in pure culture or in competition with their isogenic parent (i.e., SP_6), *in vitro* and *in vivo*.

Cost of Azm^r mutations to the growth of *C. caviae in vitro*. A single mutation in the single 23S rRNA *C. caviae* gene conferred high-level resistance to AZM, with a MIC greater than 20 μg/ml in the plaque assay, compared to 100 ng/ml for the parental *C. caviae* strain. This suggests that the mutants should have a clear growth advantage over the sensitive parent in the presence of drug. However, to be able to survive in a natural environment, a bacterium carrying a mutated allele must also compete with the wild-type ancestor bacterial population in the absence of drug. The outcome of the competition process depends on the bacterium's relative fitness, defined as the

TABLE 1. Ribosomal mutations observed in spontaneous Azm^r isolates of *C. caviae*[a]

Day (postinoculation) of recovery from animal	Wild-type clonal strain[b]	Frequency of spontaneous AZM resistance[c]	No. of isolates with mutation at indicated position in 23S rRNA gene (representative strain)			
			$A_{2058}C$	$A_{2058}G$	$A_{2059}C$	$A_{2059}G$
9	SP_1	$(4.3 \pm 7.1) \times 10^{-9}$	6 (SP_1AZ_1)	0	0	0
	SP_2	$(2.7 \pm 7.1) \times 10^{-9}$	0	0	1 (SP_2AZ_1)	0
	SP_5	10^{-9}	0	0	3 (SP_5AZ_1)	0
	SP_6	10^{-9}	1 (**SP_6AZ_1**)	0	1 (**SP_6AZ_2**)	0
12	SP_3	$(2.9 \pm 3.4) \times 10^{-8}$	23 (SP_3AZ_1)	0	0	0
	SP_4	7.7×10^{-10}	1 (SP_4AZ_1)	0	0	0
	SP_7	10^{-9}	0	1 (SP_7AZ_1)	0	0
	SP_8	$(2.0 \pm 2.8) \times 10^{-9}$	4 (SP_8AZ_1)	0	0	1 (SP_8AZ_3)
	SP_9	$(4.5 \pm 6.3) \times 10^{-9}$	0	0	1 (SP_9AZ_1)	0
	SP_{10}	2.0×10^{-9}	1 ($SP_{10}AZ_2$)	0	1 ($SP_{10}AZ_1$)	0

[a] Strains in bold were used in *in vitro* and *in vivo* studies.
[b] Infectious particles from a single plaque were expanded during three to four developmental cycles to obtain clonal populations of *C. caviae*.
[c] The frequencies of appearance of resistant mutants are relative to the total number of PFU infecting a confluent mouse fibroblast monolayer growing in a 60-mm dish ± the standard deviation.

efficiency of multiplication and transmission of the mutant cell in comparison with that of the wild-type ancestor strain (2). *C. caviae* SP_6 Azm^r variants harboring the $A_{2058}C$ (i.e., SP_6AZ_1) and $A_{2059}C$ (i.e., SP_6AZ_2) mutations in the 23S rRNA gene formed plaques in mouse fibroblast L2 monolayers that were 34% and 53% smaller, respectively, than those formed by the parent strain in the absence of drug (Table 2). Since the three *C. caviae* isolates are isogenic, apart from their rRNA mutations, this observed decrease in plaque size is directly linked to the effect of the mutations on the bacterial physiology *in vitro*.

The developmental cycle of *Chlamydia* spp. alternates between replication and growth as RBs and conversion to EBs for transmission (1). When we followed the formation of infectious particles throughout one developmental cycle in the absence of drug (Fig. 1), it was clear that both *C. caviae* Azm^r mutants were delayed in the RB-to-EB transition, as seen by their extended eclipse phase, i.e., the period in which infectious progeny EBs have differentiated into noninfectious RBs, in comparison to the level for the parent strain (Table 2). Yet, the doubling time for EB formation during the exponential phase of the development cycle was severely affected only for SP_6AZ_1 with the $A_{2058}C$ mutation, i.e., 29 min versus 21.5 and 20 min for SP_6AZ_2 with the $A_{2059}C$ mutation and the wild-type SP_6 *C. caviae* parent, respectively (Table 2). Nevertheless, EB production seemed to plateau at the same time for each of the three isolates, i.e., at about 34 h p.i. (Fig. 1). As a result, the final rate of EB production per development cycle was about 10-fold lower for the two Azm^r mutants than for the isogenic sensitive strain (Table 2).

The fitness cost conferred by the $A_{2058}C$ and $A_{2059}C$ mutations in the 23S rRNA gene was further confirmed during coinfection of wild-type *C. caviae* SP_6 with each Azm^r isogenic variant in L2 cell monolayers in the absence of selection. The competition indices (CIs) (4) ranged from 0.15 to 0.28 at the end of the first round of competition and 0.06 to 0.17 after the second round, therefore demonstrating that both mutants were clearly outcompeted by the parent strain *in vitro* (Table 2).

Cost of Azm^r mutations to the growth of *C. caviae* in single infection *in vivo*. Because the bacterial traits defining fitness in an *in vitro* situation might be different from the ones important in the natural biological niche, we studied the growth and virulence of two Azm^r *C. caviae* mutants and their isogenic parent in guinea pigs by using the ocular infection model. When groups of five guinea pigs were inoculated in both eyes with 10^4 IFU of the wild-type parent SP_6, the course of the infection closely paralleled that reported previously for the

TABLE 2. Physiological costs due to mutations in the 23S rRNA gene associated with Azm^r in *C. caviae* SP_6

C. caviae strain	23S rRNA mutation[a]	Plaque size[b] (mm)	Duration of eclipse phase (h)[c]	Rate of 2-fold EB increase (min)[c,e]	EB generation rate (fold increase)[f]	Competition index[g] at indicated no. of passages	
						One (43 h p.i.)	Two (86 h p.i.)[h]
SP_6	None (wild type)	0.98 ± 0.14	19	20	1,530 ± 427	NA	NA
SP_6AZ_1	$A_{2058}C$	0.65 ± 0.13	19–22[d]	29	113 ± 51	0.15 ± 0.03	0.06 ± 0.01
SP_6AZ_2	$A_{2059}C$	0.46 ± 0.17	22	21.6	256 ± 94	0.28 ± 0.05	0.17 ± 0.07

[a] *E. coli* numbering system.
[b] The sizes of a minimum of 34 individual plaques were determined for each strain in the absence of antibiotic at 7 days p.i. and averaged.
[c] Data obtained from Fig. 1.
[d] The eclipse phase lasted for more than 19 h but less than 22 h.
[e] The rate of 2-fold EB increase was estimated as described in Materials and Methods.
[f] The number of IFU produced at 43 h p.i. divided by the number used at the time of infection was different for each of the three *C. caviae* strains ($P < 0.0001$; *t* test).
[g] The competition index is defined as the ratio of the output mutant/wild-type ratio at the indicated time p.i. to the input mutant/wild-type ratio. NA, not applicable.
[h] Determined after a second 43-h passage.

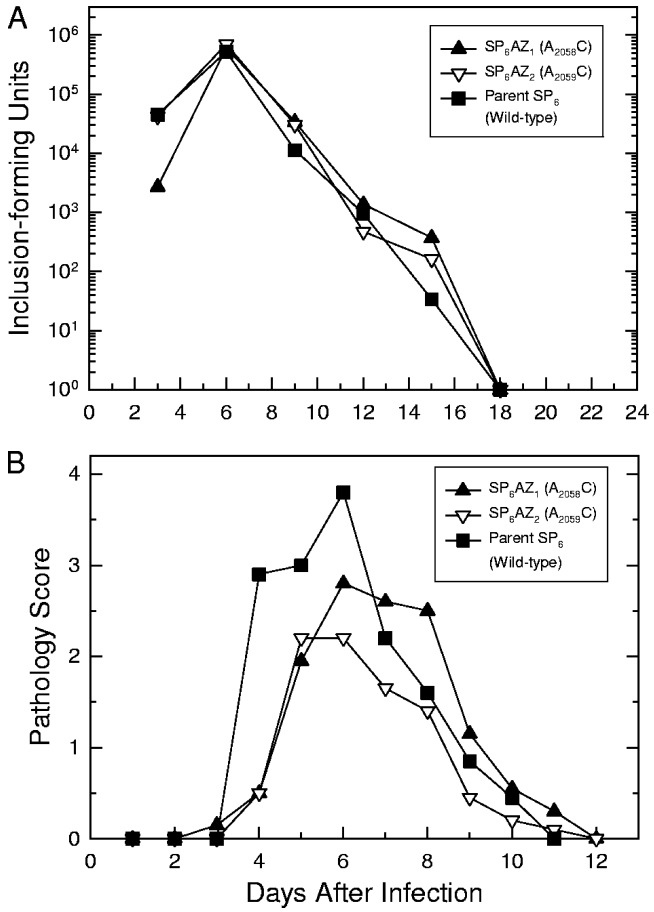

FIG. 2. Impact of AZM resistance mutations on the virulence of *C. caviae* SP_6 variants in the natural host. Ten thousand IFU from the three isogenic *C. caviae* isolates, SP_6, SP_6AZ_1 ($A_{2058}C$), and SP_6AZ_2 ($A_{2059}C$), were separately inoculated in both eyes in five animals per sample. Two independent experiments were performed and showed the same trend. The mean course of infection for one set is presented. (A) Recovery of infectious particles. Two swabs were collected every third day from the second eye. IFU were enumerated after infection onto McCoy cells from one swab, and the other was stored at −80°C. The data points represent the mean numbers of IFU for the 5 animals in the group. There were no significant differences among the 3 groups as determined by a 2-factor ANOVA with repeated measures. (B) Conjunctival pathology. Conjunctival pathology was scored every day for each eye on a scale from 0 to 4. The scores represent the means of results from 10 eyes. The scores for both eyes of an animal were always consistent, i.e., different by no more than 1 number. The pathology of animals infected with both SP_6AZ_1 and SP_6AZ_2 was significantly different from that obtained with the parent strain ($P < 0.001$), as determined by a 2-factor ANOVA with repeated measures.

wild-type polymorphic laboratory strain (Fig. 2). Infection reached a peak at about day 6 and was essentially cleared by day 18, with a resolution of the pathological response by day 12. When the Azm^r mutants were inoculated into the conjunctiva, there was no difference in the recovery of IFU after the peak of infection, nor was there a difference in the numbers of days in which the organisms were shed (Fig. 2A). This showed that the $A_{2058}C$ and $A_{2059}C$ mutations in the 23S rRNA gene had no effect on the ability of the organism to infect and grow in the natural environment. Nevertheless, the intensity of conjunctival inflammation as reflected by the pathology score (Fig. 2B) was significantly attenuated for the two Azm^r mutants in comparison to the level for the parent strain ($P < 0.001$). Indeed, while the parent SP_6 reached a maximum pathology score of 4, SP_6AZ_1 reached a maximum pathology score of 3 and SP_6AZ_2 a score of 2. This showed that the two Azm^r mutations lowered *C. caviae* virulence without affecting the ability of the bacteria to persist in the host in single infection.

Cost of Azm^r mutations to the growth of *C. caviae* in competitive infection *in vivo*. The ability of the two 23S rRNA mutants to persist in the host similarly to the wild-type strain was surprising, considering that the developmental cycles of the two mutants were affected in cell culture. Consequently, we tested SP_6AZ_1 and SP_6AZ_2 for their abilities to compete with the isogenic parent strain in the natural (guinea pig) environment. Equal numbers of mutant and wild-type bacteria were mixed and then inoculated in the conjunctiva of guinea pigs, and the infection was monitored over 18 days as described above. In general, the pathology scores in the coinoculation group were significantly higher than that obtained with the mutant alone (Fig. 3A and B), although in the coinoculation group containing SP_6AZ_1, the pathology score was still somewhat lower than that obtained with the parent strain alone (Fig. 3A). Nevertheless, these data demonstrated that the pathological response to the wild-type strain was dominant in the host. Interestingly, in contrast with the previous single infections, we observed a nearly 1-log reduction in the recovery of each mutant in the presence of the SP_6 isolate over the course of infection in comparison to the level for the parent strain in the mixture ($P < 0.001$). The competition indices (4) calculated at 6 to 12 days postinfection ranged from 0.18 to 0.02 for SP_6AZ_1 and 0.12 to 0.61 for SP_6AZ_2. Then, both mutants were cleared from the host at 15 days p.i., whereas the parent was cleared 3 days later, at 18 days p.i. (Fig. 4A and B). This showed that both Azm^r variants were clearly outcompeted by the antibiotic-sensitive parent strain in the ocular infection model in the absence of drug. Interestingly, while significantly more bacteria of the parent strain than of either mutant were recovered in the coinoculation group, the parent strain still did not grow as well as it did when inoculated into the animal alone. Consequently, the $A_{2058}C$ and $A_{2059}C$ mutations in the 23S rRNA gene, conferring resistance to AZM, bear a fitness cost to *C. caviae*'s abilities to grow in cell culture and to compete with the wild-type strain in the natural host environment.

DISCUSSION

Azithromycin is probably the most frequently used antibiotic against *Chlamydia* infections today (31), yet mutations which confer Azm^r in these obligate intracellular pathogen do not appear to be selected for *in vivo* (14, 15, 36). Of particular interest for this study, tetracycline-resistant isolates of *C. suis*, a swine strain of *Chlamydia*, started to appear in pig farms only 50 years after the introduction of chlortetracycline as a growth promoter to poultry, swine, and cattle (18). Consequently, emergence of antibiotic resistance is not an impossible scenario for chlamydiae, despite their isolated niche, which limits the opportunity for acquisition of antibiotic resistance genes from other organisms, and their particular biphasic developmental cycle, which constitutes an evolutionary bottleneck (34). We previously showed that the low number of rRNA

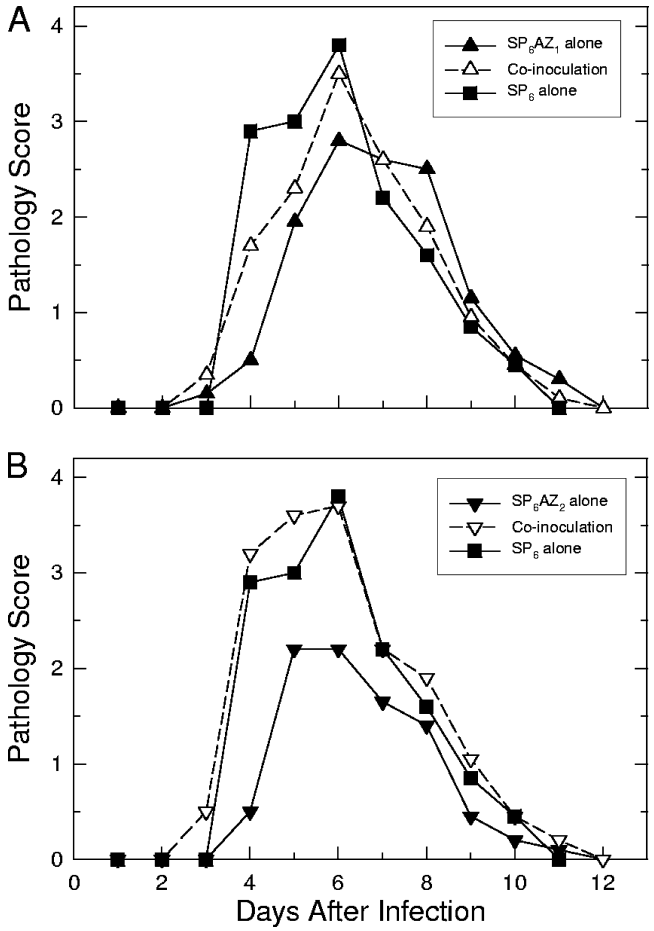

FIG. 3. Conjunctival pathology observed after single infection or coinoculation of wild-type *C. caviae* SP6 and the two 23S rRNA mutants, SP_6AZ_1 ($A_{2058}C$) and SP_6AZ_2 ($A_{2059}C$), in the natural host. Ten thousand IFU from the wild type and one Azm^r isogenic mutant of *C. caviae* were coinoculated in both eyes of five animals per sample. Conjunctival pathology was scored as described in the legend to Fig. 2. Results from the coinfection experiments are shown with dotted lines, while 12 results from the single infections (from Fig. 2B) are shown with solid lines. (A) The scores of the coinoculation group ($P < 0.008$) and the SP_6AZ_1-alone group ($P < 0.001$) were significantly different from that of the SP_6-alone group as determined by a 2-factor ANOVA with repeated measures; however, the score of the SP_6AZ_1-alone group was significantly lower than that of the coinoculation group ($P < 0.016$). (B) The score of the coinoculation group ($P < 0.008$) was not significantly different from that of the SP_6-alone group as determined by a 2-factor ANOVA with repeated measures. However, the score of the SP_6AZ_2-alone group ($P < 0.001$) was significantly lower than those of both the coinoculation group ($P < 0.001$) and the SP_6-alone group ($P < 0.001$).

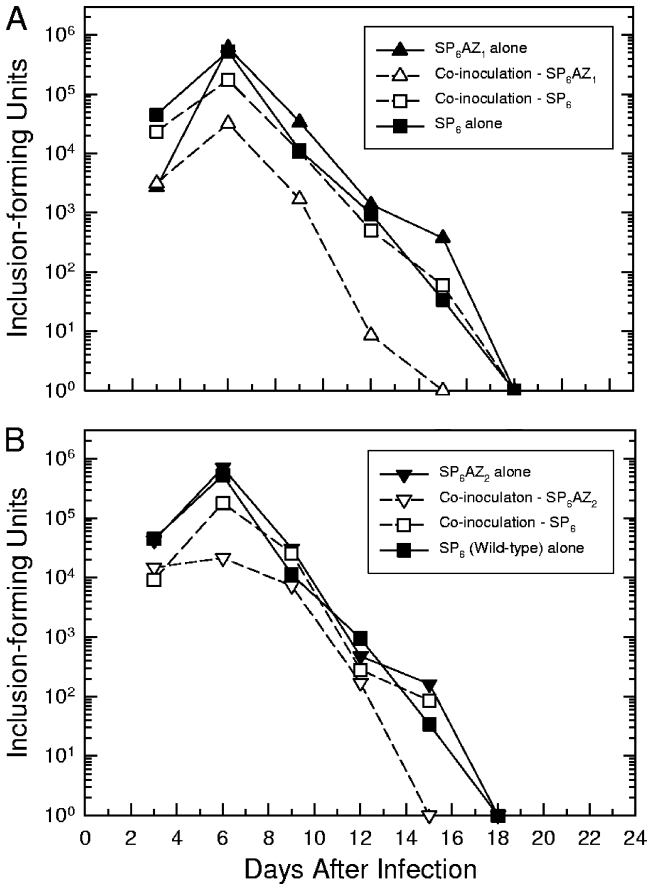

FIG. 4. Competition between wild-type *C. caviae* SP_6 and the two 23S rRNA mutants, SP_6AZ_1 ($A_{2058}C$) and SP_6AZ_2 ($A_{2059}C$), in the natural host. Coinoculation was performed as described in the legend to Fig. 3. Two swabs were collected every third day from one eye. IFU were enumerated after inoculation onto McCoy cells with (AZM IFU) and without (total IFU) AZM. The number of wild-type IFU was calculated as the total number of IFU minus the number of Azm^r IFU. Results from the coinfection experiments are shown with dotted lines, while results from the single infections (from Fig. 2A) are shown with solid lines. (A) The level of SP_6AZ_1 in the coinoculation group was significantly lower than the levels of SP_6AZ_1 alone ($P < 0.001$), SP_6 alone ($P < 0.001$), and SP_6 in the coinoculation group ($P < 0.001$) as determined by a 2-factor ANOVA with repeated measures. The level of SP_6AZ_1 alone was not significantly lower than that of SP_6 alone, but the level of SP_6 in the coinoculation group was significantly different from that of SP_6 alone ($P < 0.001$). (B) The level of SP_6AZ_2 in the coinoculation group was significantly lower than the levels of SP_6AZ_2 alone ($P < 0.001$), SP_6 alone ($P < 0.001$), and SP_6 in the coinoculation group ($P < 0.001$) as determined by a 2-factor ANOVA with repeated measures. The level of SP_6AZ_2 alone was significantly lower than that of SP_6 alone ($P < 0.02$), and the level of SP_6 in the coinoculation group was significantly different from that of SP_6 alone ($P < 0.001$).

operons in chlamydiae allows for the development of spontaneous AZM resistance *in vitro* (6). The present study is a continuation of these studies for analyzing the impacts of such mutations on bacterial physiology and virulence *in vitro* and *in vivo*, using *C. caviae*, a natural pathogen of guinea pigs, as a model.

Treatment failures (defined as *Chlamydia* persistence 1 month after treatment) following macrolide therapy have been reported for humans as well as animals, but most reports do not address the role of genetic resistance in the recurrence of chlamydial infections (15, 21, 24, 29, 36). We found that spontaneous high-level AZM resistance arose in *C. caviae* at frequencies that fall within the ones determined for *C. psittaci* 6BC or *Mycobacterium avium*, two other bacterial groups characterized by the presence of a single rRNA operon (6). Spontaneous AZM resistance occurred at frequencies ranging from 10^{-8} to 10^{-10}, depending on the clonal population of *C. caviae* isolated from the host, suggesting that significant genomic differences exist among the bacteria present in the initial labora-

tory stock. High-level AZM resistance arose in *C. caviae* following a single mutation in the single 23S rRNA gene, resulting in either an A-to-C or an A-to-G mutation at position 2058 or 2059. Such mutations have been found to confer resistance to other clinically relevant antibiotics, such as the macrolide erythromycin and the lincosamide clindamycin, in other important bacterial pathogens, including mycobacteria and *C. psittaci* 6BC (6). Note that the $A_{2058}G$ substitution in 23S rRNA, which is the most common macrolide resistance mutation encountered (10, 35), was found in none of the 30 strains with spontaneous mutations conferring Azm^r isolated previously for *C. psittaci* 6BC and in only 1 out of 45 independent mutants of *C. caviae* analyzed in this study, i.e., SP_7. It is tempting to speculate that SP_7 possesses genetic factors such as an intragenic or extragenic compensatory mutation to balance the cost of this mutation to chlamydiae (8, 20).

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., the strain's rate of infectious transmission and its ability to compete with other strains (2). When we compared the growth in cell culture of the susceptible parent to that of two isogenic *C. caviae* Azm^r variants in the absence of selection, it was clear that the $A_{2058}C$ and $A_{2059}C$ mutations in the 23S RNA gene drastically affected the transmissibility of *C. caviae*, in agreement with our previous study with *C. psittaci* 6BC (6). The rate of formation of infectious particles was about 10 times lower for the Azm^r mutants, and they were rapidly outcompeted by the parent strain in cell culture, where growth conditions had been optimized for chlamydial growth. In the more stressful natural environment of the guinea pig, both mutants were outcompeted and cleared 3 days earlier than the wild-type strain from the eyes of infected guinea pigs. Consequently, the two Azm^r *C. caviae* isolates would likely not persist in the absence of selection unless compensatory mutations are selected to adapt to the costs of chromosomal antibiotic resistance (8, 19, 20).

The processes of microbial infections are intimately linked to the immune responses of the host in its effort to resolve the infection. Although the $A_{2058}C$ and $A_{2059}G$ mutations in the 23S rRNA gene inhibited bacterial growth in cell culture, both mutants were surprisingly able to survive in the host as long as the parent in the ocular infection model: infections were self-limiting, peaked at about 6 days postinoculation, and lasted about 18 days for all three isolates, provided that the mutants were not in competition with the parent. To the best of our knowledge, such a discrepancy between growth *in vitro* and persistence in the host has never been described for any chlamydial isolates previously. The ability of the innate immune system to quickly recognize and respond to an invading pathogen is essential for controlling the infection. Clearly, survival in the host is a combination of both bacterial multiplication in the host and bacterial clearance by the host antimicrobial defense system. Growth of the two Azm^r mutants was likely affected *in vivo* too, leading to fewer infected cells and hence lower levels of chemokine/cytokine production and a weaker inflammatory response than in animals infected with the parent wild-type *C. caviae* strain, which grew faster but induced a stronger inflammatory response. As a consequence, the survival rates of the fast-growing and the slow-growing strains were similar in the host in pure infection. In coinfection experiments, it is clear that the wild-type and the Azm^r *C. caviae* isolates compete for growth and likely for nutrients, since the parent strain was not able to multiply to the same extent as when inoculated in the guinea pigs alone. Nevertheless, survival of the mutants was outbalanced by the strong host response elicited by the wild type, resulting in earlier clearance of the drug-resistant isolates. Mutations in rRNA that affect the general translational ability of the bacteria and thus the activity of multiprotein complexes such as secretion systems are, in addition, likely to increase the vulnerability of bacteria in the host.

What is responsible for *Chlamydia* pathogenesis is still a matter of debate and is likely the combination of the innate host responses and antigen-specific adaptive cellular immune responses which induce immunopathological tissue damage (28). Azm^r mutations in the *C. caviae* SP_6 isolate attenuated chlamydial virulence, as both SP_6AZ_1 and SP_6AZ_2 elicited pathological responses that were weaker than those elicited by the wild-type strain. Interestingly, there was a correlation between the reduction in the size of the plaques formed by the isolates in mouse fibroblast L2 cells and the reduction in conjunctival pathology: plaques formed by SP_6AZ_1, with the $A_{2058}C$ mutation, were bigger than those formed by SP_6AZ_2, with the $A_{2059}C$ mutation (34% and 53% the size seen for the parent SP_6, respectively), and SP_6AZ_1 was also more virulent than SP_6AZ_2 in the ocular infection model. Interestingly, a similar relationship between plaque size and virulence had previously been noted for trachoma serovariants of *C. trachomatis* (17). However, Ramsey et al. recently proposed that chlamydial virulence is linked to the bacterial replication rate and the number of infectious particles produced per developmental cycle rather than to plaque size *per se* (25).

Though Azm^r mutations at positions 2058 or 2059 of both *C. psittaci* 6BC BC_{RB} and the *C. caviae* SP_6 23S rRNA gene confer fitness costs associated with plaque size, growth in L2 mouse fibroblast cells, and presumably virulence, the $A_{2058}C$ mutation has a stronger physiological influence on *C. psittaci* 6BC than on *C. caviae* SP_6. As the overall genetic context/environment affects the fitness cost associated with drug resistance, similar resistance mutations would bear different consequences, depending on strains, serovars, or even isolates of chlamydiae. It is likely that the more frequent resistant mutants emerged in bacteria that were better equipped to handle the resistance mutation, with fewer negative consequences to the bacterial physiology, rather than in bacteria that had a higher mutation rate. Under this hypothesis, the $A_{2058}C$ Azm^r mutation would confer less fitness cost to the *C. caviae* isolate SP_3, where this mutation appeared at a frequency of about 10^{-8}, than to the isolate SP_4, where the $A_{2058}C$ mutation emerged at a much lower rate.

It is clear that many interacting factors would influence the probability of AZM resistance development in chlamydial infections. Antibiotic resistant mutants selected *in vivo* tend to be ones with little or no fitness cost *in vitro* (13, 23, 30). For chlamydial mutants to persist *in vivo*, they would have to overcome the host clearance response induced by the wild-type bacterial population. However, it is not yet clear how much of this response would be elicited during treatment. Efficient antichlamydial treatments prevent transmission, cure the infection, and prevent the development of immunopathology. Nev-

ertheless, they could also interfere with the acquisition of protective immunity, creating what has been termed an arrested immunity stage contributing to the current increases in the rates of *C. trachomatis* incidence and reinfection (9). AZM and other macrolides have additional immunomodulatory and anti-inflammatory properties that may also affect the development of a protective and/or pathological host response (32). If bacterial multiplication is essential for the acquisition of protective immunity, it would be interesting to examine if the two 23S rRNA mutants of *C. caviae* SP_6 would be able to overcome the wild-type strain in the presence of AZM in the host. These results may further improve the understanding of chlamydial therapy and biology.

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