GrgA as a potential target of selective antichlamydials

Short title: GrgA as a potential target of selective antichlamydials

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ABSTRACT

Chlamydia is a common pathogen that can causes serious complications in the reproductive system and eyes. Lack of vaccine and other effective prophylactic measures coupled with the largely asymptomatic nature and unrare clinical treatment failure calls for development of new antichlamydials, particularly selective antichlamydials without adverse effects on humans and the beneficial microbiota. We previously reported that benzal-N-acylhydrazones (BAH) can inhibit chlamydiae without detectable adverse effects on host cells and beneficial lactobacilli that dominate the human vaginal microbiota among reproductive-age women. However, the antichlamydial mechanism of BAH is not known. Whereas 4 single nucleotide polymorphisms (i.e., SNP1-4) were identified in a rare *Chlamydia* variant with a low level of BAH resistance, termed MCR, previous studies failed to establish a causal effect of any particular SNP(s). In the present work, we performed recombination to segregate the four SNPs. Susceptibility tests indicate that the R51G GrgA allele is both necessary and sufficient for the low level of BAH resistance. Thus, the *Chlamydia*-specific transcription factor GrgA either is a direct target of BAH or regulates BAH susceptibility. We further confirm an extremely low rate of BAH resistance in *Chlamydia*. Our findings warrant exploration of GrgA as a therapeutic and prophylactic target for chlamydial infections.

INTRODUCTION

Chlamydiae are important and widespread pathogens. *Chlamydia trachomatis* is a leading infectious cause of blindness in many underdeveloped countries [1]. Globally, *C. trachomatis* is the leading sexually transmitted bacterial pathogen with an estimated prevalence of 4.2% among women aged 15-49 years [2]. In the United States, there has been a steep and sustained increase in sexually transmitted *C. trachomatis* infection in the past five years; 1.7 million cases were diagnosed in 2017, which represents a 22% increase from 2013, and accounts for 60% of cases of infections reported to the Centers for Disease Control and Prevention [3]. Genital *C. trachomatis* infection in women often leads to serious complications including infertility, pelvic inflammatory syndrome, abortion or premature birth and ectopic pregnancy [4].

C. pneumoniae is another common human pathogen, which causes bronchiolitis and pneumonia. Children, young adults and elderlies are at increased risks [5]. Several *Chlamydia* species are major health threats to livestock, and are also zoonotic pathogens [6, 7]. *C. muridarum* is a useful organism that models *C. trachomatis* infection in mice [8, 9].

Chlamydiae are susceptible to several broad-spectrum antibiotics. Human chlamydial infections are clinically treated with either azithromycin or doxycycline [10]. Due to a lack of vaccine, mass azithromycin administration has been used in Africa to treat eye infection and cut off the transmission. However, this chemical prevention strategy is only partially effective [11, 12]; furthermore, it has been linked to resistance development in standing-by pathogens [13, 14].

There are at least three additional concerns for current antichlamydial therapies. First, because of their broad-spectrums, they may cause dysbiosis in the genital tract and other systems [15-17]. Whereas loss of protective lactobacilli from the vagina of reproductive-age women may increase the risk of vaginal yeast infection [17], antibiotic-induced shift of gut microbiota may

lead to problems ranging from severe diarrhea to increased risks for serious but not immediately noticeable metabolic changes [18, 19]. Second, although in culture *C. trachomatis* is highly susceptible to the therapeuticals, clinical treatment failure, which leads to persistent infection, is not rare [20, 21]. Finally, given the fact that tetracycline resistance has become widespread in *C. suis* due to farmers' use of tetracycline as a growth promoter [22-24], antibiotic resistance could emerge in other *Chlamydia* species including *C. trachomatis* and *C. pneumoniae*.

For the above-mentioned reasons, it is important to identify new antichlamydial leads, particularly selective antichlamydial leads without adverse effects on either the host or other microbes, and identify their antichlamydial mechanisms. We have reported benzal-N-acylhydrazones (BAH) as novel antichlamydial leads capable of inhibiting all three *Chlamydia* species tested, *C. trachomatis, C. pneumoniae* and *C. muridarum* [25]. Significantly, at concentrations above minimal inhibition concentrations, BAH have no adverse effects on animal cells or vaginal lactobacilli [25]. Another attractive feature of BAH is their extremely low spontaneous mutation rates leading to resistance [25, 26]. Although a *C. muridarum* variant termed MCR with a low-level of BAH resistance was initially isolated following a lengthy selection process, multiple repeated attempts to isolate additional resistant variants from mutagenized as well as non-mutagenized stocks of *C. muridarum* and *C. trachomatis* were unsuccessful [25, 26].

How BAH inhibit chlamydiae remains unknown. Compared to the parental *C. muridarum*, MCR carries four single nucleotide polymorphisms (i.e., SNP1-4) in its genome (Table 1). SNP1 causes an A228V substitution in the major outer membrane protein (MOMP). Although A228 is conserved in MOMP in *C. muridarum* and *C. trachomatis*, V228 is found in *C. pneumoniae*, which remains highly susceptible to BAH [25]. SNP2 is located at the 10th position of the 5'

85 untranslated region of the mRNA for Npt1 (ATP/ADP translocase), and is associated with a
86 decreased Npt1 mRNA level. BAH have no effect on Npt1-mediated ATP transportation,
87 suggesting that Ntp1 is unlikely a target of BAH [25]. SNP3 causes premature translation
88 termination of TC0412, a homolog of the putative virulence factor CT135 in *C. trachomatis.* The
89 truncated TC0412 contains only the N-terminal 23 amino acids, compared to the full length 365
90 amino acids. Given the hypermutable nature of *tc0412* [27] and the ultralow spontaneous BAH
91 resistance rate, TC0412 is also unlikely a BAH target. Indeed, isogenic CT135 mutants are as
92 susceptible to BAH as wild-type *C. trachomatis* [25]. SNP4 causes an R51G substitution in a
93 *Chlamydia*-specific transcription activator termed GrgA. Whereas the transcription activation
94 activity of GrgA is reduced by the substitution, it is not directly affected by BAH compounds
95 [25]. Taken together, previous biochemical studies have failed to establish a role for MOMP,
96 Npt1, TC0412 or GrgA in BAH-mediated *Chlamydia* inhibition. In this work, we establish
97 through genome recombination that the rare R51G mutation in GrgA is both necessary and
98 sufficient for BAH resistance in MCR. Our studies indicate GrgA as a promising target for
99 selective antichlamydials.
100
101 **Table 1. Terminology for SNPs in MCR**

	Effect	General term	Wild-type allele	Mutant allele
SNP1	A228V MOMP	S1(MOMP)	S1(wtMOMP)	S1(A228V MOMP)
SNP2	Decreased Npt1 mRNA	S2(Npt1)	S2(wtNpt1)	S2(d_Npt1)
SNP3	Truncated TC0412	S3(TC0412)	S3(wtTC0412)	S3(t_TC0412)
SNP4	R51G GrgA	S4(GrgA)	S4(wtGrgA)	S4(R51G GrgA)

102
103

Materials and Methods

Chlamydia strains

Parental strains used for generation of recombinant chlamydiae as well as their precursors are listed in Table 2. Wild-type *C. muridarum* MoPn and the BAH-resistant variant MCR have been described previously [25]. MoPn_Rif^R, MoPn_Spc^R, MCR_LBM^R and MCR_Rif^R were derived by culturing MoPn and MCR in medium containing appropriate inhibitors (i.e., rifampin, spectinomycin or LBM415) at gradually increased concentrations starting at sub-MIC, as we previously outlined [25, 26, 28, 29].

Table 2. Strain information

Strain	MIC			Genome base change	Mutation effect	Source and/or Reference
	Rifampin (μg/ml)	LBM415 (nM)	Spectinomycin (μg/ml)			
MoPn	0.008	32	50	NA	NA	ATCC; [25, 30]
MoPn_Rif^R	0.050	32	ND	c707469g g707471a	Q455Y RpoB	This study
MoPn_Spc^R	ND	ND	>150	c135035t	16S rRNA	This study
MCR	0.008	32	50	g59134a c399603g t472827g g935223c	A228V MOMP d_Npt1 t_TC0412 R51G GrgA	[25]; Table 1
MCR_LBM^R	0.008	>100	ND	g59134a c399603g t472827g g935223c a758622c	A228V MOMP d_Npt1 t_TC0412 R51G GrgA *def*A promoter	This study
MCR_Rif^R	>0.3	ND	50	g707450t	S476Y RpoB	This study

Positions of SNPs in the genome are based on GenBank accession no. NC_002620.

Abbreviation: MIC, minimal inhibitory concentration; NA, not applicable; Rif, rifampin; RpoB, RNA polymerase β subunit gene; Spc, spectinomycin; MOMP, major outer

membrane protein; d_Npt1, decreased Npt1 (ATP/ADP translocase) expression; t_TC0412, truncated TC0412 protein with amino acids 1-23 (missing 24-365); *def*A, peptide deformylase gene; ND, not determined.

Generation of recombinant chlamydiae

Mouse L929 cells grown in T25 flasks were coinfected with 2 parental strains at an MOI (multiplicity of infection) of 1 IFU per cell for each strain, cultured with medium containing 1 µg/ml cycloheximide. After a passage without antibiotics, they were cultured with 6 ng/ml rifampin plus 25 nM LBM415 or 6 µg/ml spectinomycin (di selection) for 6 passages. 90 µM CF0001 was included as part of tri selection either following the completion of or in parallel to the di selection for 6 passages.

Generation of clonal populations

Clonal populations of parental strains with resistance to rifampin, spectinomycin or LBM415 (Table 2) and recombinant chlamydiae were obtained mostly by limiting dilution [31] and in several cases by plaquing [32] following published protocols. When using limiting dilution, EB stocks were diluted to approximately 1 IFU per 96-well plate.

Genotyping

Genomic DNA was prepared from infected cells using a Quick-gDNA™ MiniPrep kit (Zymo). DNA fragments for genes of interest were PCR-amplified, and sequenced at Genscript or MacrogenUSA using primers listed in Table S1 [25]. Peaks of sequencing chromatograms were manually checked for evidence for coexistence of wild-type and mutant alleles.

141

142 **Comparative BAH susceptibility tests**

143 Near confluent HeLa cells were inoculated with chlamydiae at an MOI of 1 IFU per 10-30
144 cells, and cultured with medium containing 60 µM CF0001, indicated concentration of SF3,
145 control solvent DMSO (final concentrations: 1.0% for CF0001 and 1.2% for SF3) and
146 cycloheximide (1 µg/ml). 24 h later, cultures were harvested, and recoverable EB were
147 quantified as previously described (22,23).

148

RESULTS

S4(R51G GrgA) is necessary for BAH resistance

To identify a particular SNP(s) that are necessary and/or sufficient for BAH resistance, we set out to segregate the 4 SNPs through genome recombination [33-35]. To enrich recombinant chlamydiae, we first derived a rifampin-resistant variant, termed MoPn_Rif^R from wild-type *C. muridarum* strain Nigg II (traditionally referred as strain mouse pneumonitis, MoPn), and an LBM415-resistant MCR variant, termed MCR_LBM^R (Table 2). Sequencing analyses revealed that the rifampin resistance in MoPn_Rif^R was due to two base changes in a single codon of the *rpo*B gene, resulting in an amino acid substitution (Q455Y) in the β subunit of the RNA polymerase (RpoB), whereas LBM415 resistance in MCR_LBM^R was due to a single point mutation in the promoter region of the *def*A (coding for peptide deformylase) [29], resulting in the generation of a predicted stronger -35 promoter element. As shown in Fig. S1, these mutations do not affect the antichlamydial effects of CF0001 [(*E*)-*N*'-(3,5-dibromo-4-hydroxybenzylidene)-3-dinitrobenzohydrazide], a prototype BAH.

We performed two MoPn_Rif^R X MCR_LBM^R recombination studies. For the first one, we coinfected 5 flasks of L929 cells with the two parental strains, and maintained the flasks as independent lines (W1-5) in subsequent passages (Fig. 1A). We selected for recombinant chlamydiae using sub-minimal inhibitory concentrations of rifampin and LBM415 (see experimental procedures). At the end of the 6th passage of the Rif/LBM di selection, Sanger's sequencing revealed that wild-type *rpo*B and *def*A alleles were apparently eliminated in 4 of the 5 lines (Fig. 1B), whereas the W4 line still retained both the wild-type and mutant alleles of *rpo*B and *def*A. These results indicate that at this point the W1, W2, W3 and W5 lines were comprised

of recombinants and very few (if any) parental organisms (Fig. 1B). Contrast to the *rpo*B and *def*A selection markers, almost all loci of the 4 SNPs displayed a mixture of wild-type and mutant alleles in these lines (Fig. 1B), suggestive of good recombination complexity at most of the SNP loci. Exceptions were apparent absence of S1(A228V MOMP) and S2(wtNpt1) alleles in the W1 and W3 lines, respectively (Fig. 1B), likely reflecting low recombination complexity at these sites in these lines. We then continued the selection for BAH resistance by adding CF0001 to the Rif/LBM di selection for 6 additional passages. Interestingly, by the end of the 6th passage with the Rif/LBM/CF tri selection, we observed apparent elimination of wild-type alleles at all the 4 SNP loci in all 5 lines (Fig. 1C), even for the locus of S1(MOMP), where the S1(A228V MOMP) allele was unnoticeable (and thus must be present at a very low percentage) prior to the start of the tri selection (Fig. 1B).

Fig. 1. The Rif/LBM di selection is largely non-discriminatory towards either allele at the SNP loci, but the Rif/LBM/CF tri selection eliminates wild-type alleles. (A) Schematic presentation of genomes of MoPn_Rif^R (*C. muridarum* MoPn variant resistant to rifampin) and MCR_LBM^R (derivative of MoPn variant MCR with resistance to LBM415) and experimental flow for recombination, sequential di and tri selection, genotyping, susceptibility tests and data presentation. (B) Genotyping results of Rif/LBM-selected populations from the first MoPn_Rif^R X MCR_LBM^R recombination study. (C) Genotyping results of Rif/LBM/CF-selected populations showing elimination of wild-type alleles at all SNP loci by CF0001 from Rif/LBM-selected populations in (B). (D) Experimental flow for MoPn_Rif^R X MCR_LBM^R recombination, parallel di and tri selection, genotyping, susceptibility tests and data presentation. (E) Genotyping results of

195 Rif/LBM-selected populations of the second MoPn_Rif^R X MCR_LBM^R recombination
196 study. (F) Genotyping results of Rif/LBM/CF-selected populations obtained in parallel to
197 those in (E). (A, D) S1, S2, S3 and S4 signify the four SNPs. Rif, rifampin; LBM,
198 LBM415; CF, CF0001; 6p, 6 passages. (B, C, E, F) Green "W" and red "M" signify a
199 wild-type allele and a mutant allele, respectively, at the SNP loci. Green "s" and red "r"
200 signify wild-type and mutated genotypes, which render susceptibility and resistance,
201 respectively, to either rifampin or LBM415.
202
203 For the second recombination study, we co-infected 3 flasks of L929 cells with MoPn_Rif^R
204 and MCR_LBM^R. We modified the selection protocols by splitting each line into two fractions,
205 and subjecting them to the Rif/LBM di selection and Rif/LBM/CF tri selection in parallel (as
206 opposed to sequential di and tri selections above) (Fig. 1D). By the end of 6th passages with
207 either selection regimen wild-type *rpo*B and *def*A alleles were apparently absent, indicating
208 (near) complete elimination of parental chlamydiae (Fig. 1E). In Rif/LBM-selected populations,
209 both wild-type and mutant alleles were present at most of the SNP loci (Fig. 1E). In comparison,
210 Rif/LBM/CF-selected populations exhibited only mutant alleles at all the loci (Fig. 1F). The
211 consistent elimination of chlamydiae carrying any wild-type alleles by CF0001 in both
212 recombination studies suggests two alternative probabilities. In one, all of the 4 mutant alleles in
213 MCR are necessary for BAH resistance. In the other, only 1 or up to 3 of the 4 mutant alleles are
214 necessary, but accompanying mutant allele(s) compensate for growth disadvantages caused by
215 the mutant allele(s) required for BAH resistance.
216 To distinguish between these probabilities, we set out to generate clonal populations from the
217 W1, W2, W3 and W5 lines that were subjected to 6 passages of Rif/LBM di selection through

either limiting dilution [31] or plaquing [32]. A total of 79 clonal populations were generated. Complete genotyping analyses were performed for 32 populations, which represented only 8 of the 16 possible recombinant genotypes (Table S2). The remaining 47 clonal populations were only partially genotyped because initial sequencing data indicated that either they were likely redundant populations or their genotypes were considered unhelpful based on BAH susceptibility data that were already obtained from fully genotyped populations.

BAH susceptibility tests were performed for 14 clonal populations, which represented all of the 8 defined unique genotypes, alongside with MCR and MoPn (Fig. 2). As expected, both all-wild-type allele populations tested (i.e., w5c2 and w1c15) displayed susceptibility to CF0001 as wild-type MoPn, whereas both all-mutant-allele populations tested (i.e., w3c2 and w5c4) displayed resistance as MCR. Interestingly, among 10 clonal populations with 1-3 mutant alleles, only w2c10, the sole clonal population with an S4(R51G GrgA) allele, was resistant, whereas all 9 other populations, which carried S4(wtGrgA) were susceptible even though they had up to 3 mutant alleles at S1(MOMP), S2(Npt1) and/or S3(TC0412). These results suggest that among the 4 SNPs in MCR, only S4(R51 GrgA) is required for BAH resistance. However, due to the coexistence of the S3(t_TC0412) allele in w2c10, and the lack of a population with S4(R51G GrgA) as the only mutant allele, it was unclear whether the R51G GrgA allele alone is sufficient for BAH resistance.

Fig. 2. S4(R51G GrgA) is necessary for BAH resistance.

CF0001 inhibition profile of representative fully genotyped clonal populations. Green "W" and red "M" signify a wild-type allele and a mutant allele, respectively. EB

240 formation was determined in medium containing either 60 µM CF0001 or 1% DMSO as
241 vehicle control. Results were averages ± standard deviation of triplicate experiments.
242
243 **SNP4(R51G GrgA) is sufficient for BAH resistance**
244 In the above MoPn_Rif^{R} X MCR_LBM^{R} recombination studies, the selection markers *rpo*B
245 and *def*A are both located between S3(TC0412) and S4(GrgA) in the MoPn genome (Fig. 1A,
246 D). To obtain variants with a genotype of S1(wtMOMP), S2(wtNpt1), S3(wtTC0412) and
247 S4(R51G GrgA), we decided to use two selection markers separated by a SNP(s). We derived a
248 spectinomycin-resistant *C. muridarum* variant (MoPn_Spc^{R}), which carries a single point
249 mutation in the 16S rRNA (Table 3). Unlike *rpo*B and *def*A, the 16S rRNA gene is located
250 between S1(MOMP) and S2(Npt1) (Fig. 3A). This mutation did not affect inhibition of
251 chlamydiae by CF0001 (Fig. S1).
252 Initially, we tried to generate but failed to select for MoPn_Spc^{R} X MCR_LBM^{R}
253 recombinants using the Spc/LBM (spectinomycin plus LBM415) selection regimen in 3 different
254 attempts. In the regimen, the concentration of LBM415 was either the same as or higher than the
255 concentration used for the above Rif/LBM regimen. The Spc/LBM regimen failed to eliminate
256 wild-type *def*A allele although it successfully eliminated wild-type 16S rRNA. These findings
257 suggest that spectinomycin and LBM415 are incompatible for recombinant selection.
258 We next derived an MCR variant with rifampin resistance (MCR_Rif^{R}). Similar to
259 MoPn_Rif^{R}, which expresses a Q455Y RpoB, MCR_Rif^{R} expresses an S476Y RpoB (Table 3).
260 MCR_Rif^{R} retained low level of CF0001-resistance as MCR and MCR_LBM415 (Fig. S1).
261 We created 6 independent MoPn_Spc^{R} X MCR_Rif^{R} recombinant lines. Each of the 6 lines
262 was subjected to parallel Spc/Rif di selection and Spc/Rif/CF tri selection, and subsequently to

263 genotyping analyses (Fig. 3A). After 6 passages with either selection, most (if not all) parental
264 chlamydiae were eliminated, as indicated by apparent lack of the spectinomycin-susceptible 16S
265 rRNA allele and rifampin-susceptible *rpo*B allele (Fig. 3B, C).
266
267 **Fig. 3. S4(R51G GrgA) is sufficient for BAH resistance.**
268 (A) Schematic presentation of genomes of MoPn_Spc^R (*C. muridarum* MoPn variant
269 resistant to spectinomycin) and MCR_Rif^R (derivative of MoPn variant MCR with
270 resistance to rifampin) and experimental flow for recombination, recombinant selection,
271 genotyping, susceptibility tests and data presentation. (B) Genotyping results of Spc/Rif-
272 selected populations. (C) Genotyping results of Spc/Rif/CF-selected populations. (D)
273 CF0001 inhibition profiles of representative fully genotyped clonal populations as
274 determined in Fig. 2. (A-D) Refer to Fig. 1 legend for signification and abbreviations.
275
276 Genotyping analyses suggest that allele diversity for the 4 SNPs in the Spc/Rif-selected
277 MoPn_Spc^R X MCR_Rif^R recombinants (Fig. 3B) was lower than that of the Rif/LBM-selected
278 MoPn_Rif^R X MCR_LBM^R recombinants (Fig. 1B). Although the Spc/Rif di selection displayed
279 no bias for either S1(MOMP) alleles, it showed a consistent bias for the S2(wtNpt1) in all 6
280 lines. It also displayed bias for the S3(t_TC0412) alleles in 3 (r1-3) of the 6 lines. For the
281 S4(GrgA) locus, it displayed bias for (in lines r1 and r3) and against (in line r6) the S4(R51G
282 GrgA) allele, and no apparent bias in the remaining 3 lines (r2, r4 and r5).
283 In a striking contrast to the Rif/LBM/CF-selected MoPn_Rif^R X MCR_LBM^R recombinants,
284 which consistently carried only mutant alleles at all 4 SNP loci (Fig. 1C), Spc/Rif/CF-selected
285 MoPn_Spc^R X MCR_Rif^R recombinants contained both S1(wtMOMP) and/or S3(wtTC0412)

alleles, in addition to mutant alleles, at the S1(MOMP) and S3(TC0412) loci, but only wild-type allele at the S2(Npt1) locus (Fig. 3C). The only consistency between Rif/LBM/CF-selected populations and Spc/Rif/CF-selected populations is the lack of wild-type allele at the S4(GrgA) locus (Fig. 3C), further supporting the notion that the S4(R51G GrgA) allele is necessary for BAH resistance (Fig. 2).

We generated 21 clonal populations from Spc/Rif-selected populations, and 13 clonal populations from Spc/Rif/CF-selected populations. These 34 clonal populations represented 6 of the 16 possible recombinant genotypes (Table S3). 12 representative clonal populations were tested for BAH resistance alongside with MCR and MoPn. All 4 clonal populations carrying the S4(wtGrgA) allele demonstrated susceptibility to CF0001, whereas all 8 clonal populations carrying the S4(R51G GrgA) allele including the 3 populations (r8s6, r8s7 and r8s11) carrying S4(R51G GrgA) and wild-type alleles for the 3 remaining SNP loci were resistant (Fig. 3D). These findings, together with data presented in Fig. 2, indicate that the S4(R51G GrgA) allele is both necessary and sufficient for BAH resistance, which can be viewed more clearly by arranging all phenotypically characterized clonal populations from both the MoPn_Rif^R X MCR_LBM^R recombination and the MoPn_Spc^R X MCR_Rif^R recombination by their S4(GrgA) genotype (Table S4).

Ultralow rate of spontaneous BAH resistance

Previous studies indicated that BAH resistance in chlamydiae occurs at extremely low rates. The observations that the Rif/LBM/CF selection consistently eliminated wild-type S1(wtMOMP), S2(wtNpt1) and S3(wtTC0412) (Fig. 1C, F) even though S4(R51G GrgA) is the only mutant allele that is required for BAH resistance suggest that accompanying mutations help

the survival of chlamydiae carrying S4(R51G GrgA) in the presence of BAH on the background of mutated *rpo*B and *def*A. We next determined whether presence of mutant alleles at the S1(MOMP), S2(Npt1) and S3(TC0412) loci in the genome helps selection for variants with GrgA mutation conferring BAH resistance by using the clonal population w3c5, which carries wild-type GrgA allele at the S4(GrgA) locus but mutant alleles at all three remaining SNP loci.

A total of 6 screens were carried out with w3c5. The first 2 screens were initiated with a combined 0.9 X 10^7 inclusion-forming units (IFU) of non-mutagenized elementary bodies (EB) and selection was carried out with CF0001 (gradually increased from 80-120 µM) as a sole selection agent. The second 2 screens were initiated with the same number of non-mutagenized EB but selection was carried out with the Rif/LBM/CF tri selection regimen that was used to select for CF0001-resistant recombinants (Fig. 1A, D). No resistant chlamydiae emerged with either selection regimen.

The final two screens for CF0001-resistant variants were initiated with EBs prepared from cultures treated with 2 or 5 mg/ml ethyl methanesulfonate, a DNA-damaging reagent that has been used to mutagenize chlamydiae previously [29, 36, 37]. We also failed to obtain resistant chlamydiae in each of these attempts starting with 2 X 10^7 IFUs of EB. The repeated failures to isolate additional CF0001-resistant variants suggest that only very few and specific mutations in GrgA can lead to BAH resistance and/or sustain chlamydiae.

S4(R51G GrgA)-mediated BAH resistance is overcome by SF3

Compared to the prototype antichlamydial BAH CF0001, SF3 [(*E*)-*N*'-(3,5-dibromo-4-hydroxybenzylidene)-3,5-dinitrobenzohydrazide], a recently developed BAH, has a stronger antichlamydial activity, and can fully inhibit MCR [26]. At 80 µM, SF3 also achieved full

inhibition of all three tested clonal populations (r8s6, r8s7 and r8s11) with S4(R51G GrgA) as the sole mutant allele although at lower concentrations it inhibited the wild-type MoPn more efficiently (Fig. 4). These results indicate that S4(R51G GrgA)-mediated BAH resistance can be overcome by SF3.

Fig. 4. Complete inhibition of clonal populations with S4(R51G GrgA) as the sole mutant allele by 80 µM SF3.

Results are averages ± standard deviations of duplicate experiments. Nd, none detected. Double asterisks signify statistically significantly higher number of EBs formed by the clonal recombinant populations, as compared to wild-type MoPn, in the presence of 20 or 40 µM SF3 ($p < 0.01$, 2-tailed *t* test).

DISCUSSION

BAH belong to a novel group of selective antichlamydials [25, 26]. The genome of the rare BAH-resistant *C. muridarum* variant MCR carries four SNPs [25]. Through extensive genetic analyses and susceptibility tests reported here, we have unequivocally established that the S4(R51G GrgA) allele is both necessary and sufficient for a low level of BAH resistance.

GrgA functions as a transcription activator. Although it has been previously demonstrated that BAH is incapable of blocking the transcription activation activity of GrgA *in vitro* [25], it is possible that BAH functions as "prodrug"; host cell- or chlamydia-derived BAH derivatives may interact with GrgA. Alternatively, BAH may interfere with another yet-to-be defined critical process involving GrgA. It is also conceivable that GrgA regulates BAH susceptibility without directly interacting with BAH or their bioactive derivatives.

Chlamydia encodes 3 sigma factors, including the major sigma factor σ^{66} and two alternative sigma factors σ^{28} and σ^{54}. As part of the RNA polymerase, the sigma factors recognize different promoter sequences. Studies have shown that GrgA activates both σ^{66}-dependent transcription and σ^{28}-dependent transcription *in vitro*, suggestive of critical roles for GrgA in chlamydial gene expression [38, 39]. Thus, GrgA is a promising candidate therapeutic and prophylactic target even if it may not be the receptor of BAH or their bioactive derivatives. GrgA is a *Chlamydia*-specific protein. Whereas it is conserved by all *Chlamydia* species, it is not found in any other organisms. Therefore, targeting GrgA will provide intrinsically high selectivity.

Previous studies have shown that random mutation rates leading to BAH resistance is extremely low in *C. trachomatis* and *C. muridarum*. Therefore, MCR represents a rare variant with only a low level of resistance. Consistent elimination of wild-type alleles at the loci of SNP1-3 from Rif/LBM/CF-selected populations suggests that the co-existence of these mutant

366 alleles helps survival of chlamydiae carrying the S4(R51G GrgA) allele in the presence of BAH,
367 rifampin and LBM415. Our failures to isolate additional BAH-resistant mutants on the
368 background of S1(A228V MOMP), S2(d_Npt1) and S3(t_TC0412) from clonal population w3c5
369 in multiple attempts with different selection regimen suggest that very few and specific GrgA
370 mutations can cause BAH resistance and/or sustain chlamydial growth.

371 Compared to the prototype antichlamydial BAH CF0001, the recently-developed SF3 has a
372 stronger antichlamydial activity while maintaining non-toxicity to mammalian cells and vaginal
373 lactobacilli [26]. It has been shown previously that MCR can be inhibited completely by SF3
374 even though it is less susceptible than wild-type MoPn to lower concentrations of SF3 [26].
375 While it is expected that clonal recombinant populations carrying S4(R51G GrgA) as sole
376 mutant allele demonstrate the same properties as MCR, these clonal populations will be more
377 useful for identifying additional selective antichlamydials that interferes with a process involving
378 GrgA.

379 In summary, we have unequivocally established that R51G GrgA is both necessary and
380 sufficient for the low level of BAH resistance in the *Chlamydia* variant MCR. These findings and
381 the facts that GrgA is a *Chlamydia*-specific protein and plays important roles in chlamydial
382 transcription indicate GrgA as a promising selective therapeutic/prophylactic target, even though
383 it is unclear whether GrgA is a direct target of BAH or regulates BAH susceptibility without
384 directly interacting with BAH. In addition to the high selectivity, the ultralow rate of BAH
385 resistance in chlamydiae is another super attractive feature for developing BAH compounds as
386 therapeutic/prophylactic agents.

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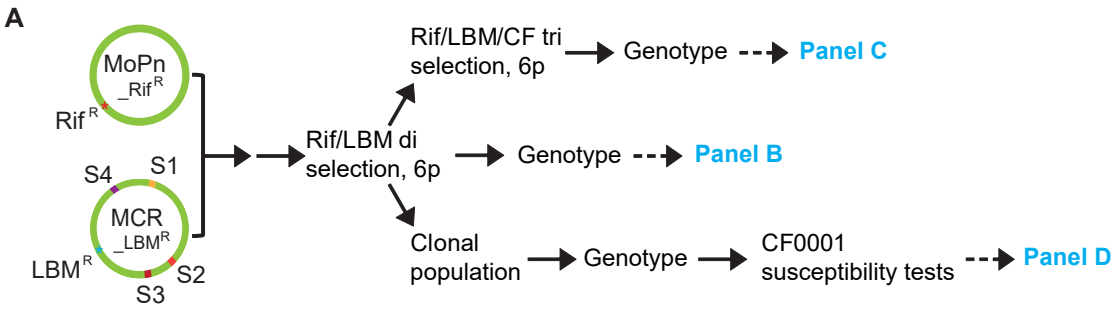
527

Fig. 1

A



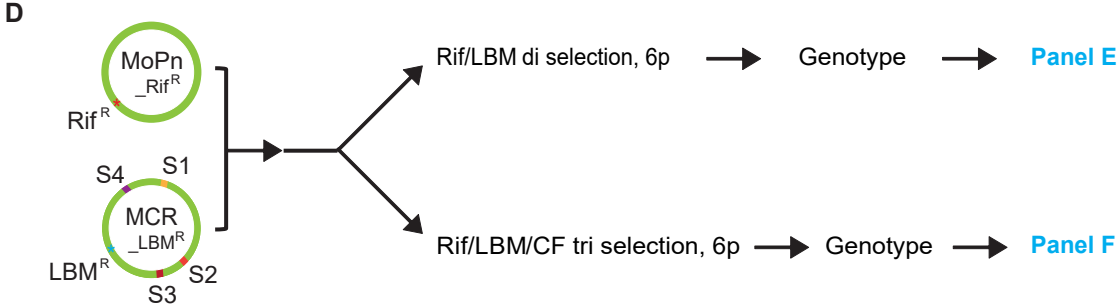
B

	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA	Rif	LBM
W1	W	MW	MW	MW	r	r
W2	MW	MW	MW	MW	r	r
W3	MW	M	MW	MW	r	r
W4	MW	MW	MW	MW	rs	rs
W5	MW	MW	MW	MW	r	r

C

	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA	Rif	LBM
W1	M	M	M	M	r	r
W2	M	M	M	M	r	r
W3	M	M	M	M	r	r
W4	M	M	M	M	r	r
W5	M	M	M	M	r	r

D



E

	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA	Rif	LBM
W6_d	MW	MW	MW	MW	r	r
W7_d	M	M	MW	MW	r	r
W8_d	MW	MW	MW	MW	r	r

F

	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA	Rif	LBM
W6_t	M	M	M	M	r	r
W7_t	M	M	M	M	r	r
W8_t	M	M	M	M	r	r

Fig. 2

Clone	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA
MCR	M	M	M	M
w3c2	M	M	M	M
w5c4	M	M	M	M
w3c5	M	M	M	W
w3c4	M	M	M	W
w1c4	M	M	W	W
w1c10	M	M	W	W
w1c13	M	W	W	W
w2c4	W	M	M	W
w2c2	W	M	M	W
w1c12	W	M	M	W
w2c10	W	W	M	M
w1c3	W	W	M	W
w5c2	W	W	W	W
w1c15	W	W	W	W
MoPn	W	W	W	W

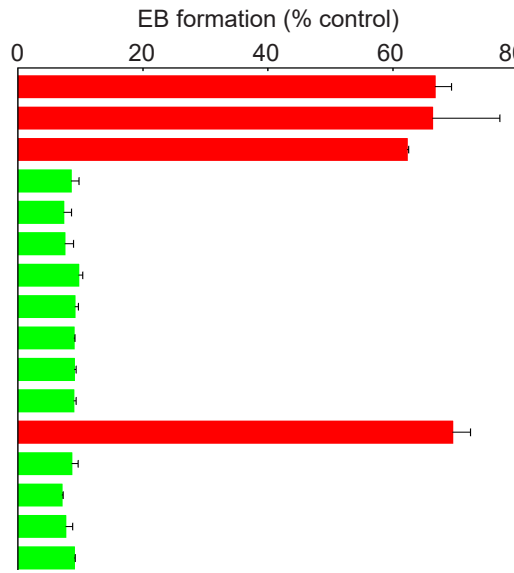
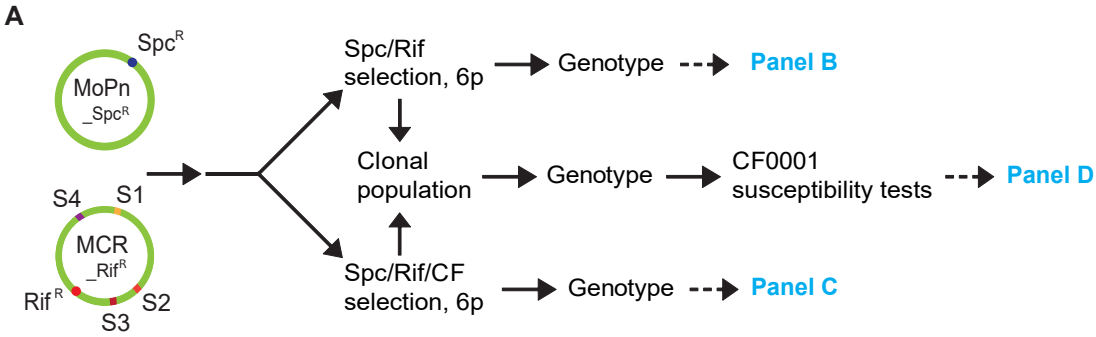


Fig. 3



B

	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA	Spc	Rif
r1	MW	W	M	M	r	r
r2	MW	W	M	MW	r	r
r3	MW	W	M	M	r	r
r4	MW	W	MW	MW	r	r
r5	MW	W	MW	MW	r	r
r6	MW	W	MW	W	r	r

C

	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA	Spc	Rif
r7	MW	W	MW	M	r	r
r8	MW	W	MW	M	r	r
r9	MW	W	M	M	r	r
r10	MW	W	M	M	r	r
r11	MW	W	MW	M	r	r
r12	W	W	MW	M	r	r

D

Clone	SNP1 MOMP	SNP2 Npt1	SNP3 TC0412	SNP4 GrgA
MCR	M	M	M	M
r8s5	M	W	M	M
r1s4	M	W	M	M
r5s1	M	W	W	M
r4s13	W	W	M	M
r4s12	W	W	M	M
r4s11	W	W	M	W
r5s2	W	W	M	W
r8s6	W	W	W	M
r8s7	W	W	W	M
r8s11	W	W	W	M
r4s9	W	W	W	W
r5s7	W	W	W	W
MoPn	W	W	W	W

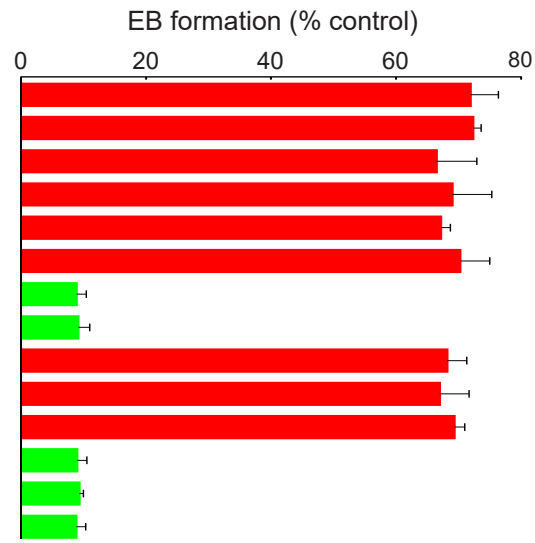
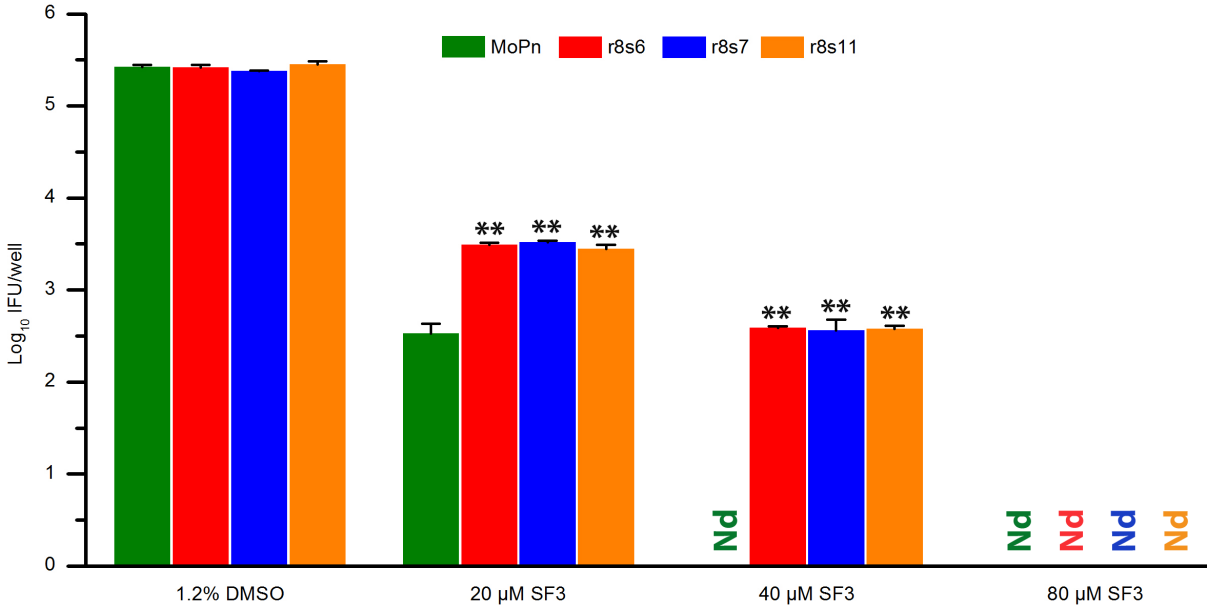


Fig. 4


MoPn
r8s6
r8s7
r8s11
Log10 IFU/well
0
1
2
3
4
5
6
**
**
**
**
**
**
Nd
Nd
Nd
Nd
1.2% DMSO
20 μM SF3
40 μM SF3
80 μM SF3