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A Strain-Specific Catalase Mutation and Mutation of the Metal-Binding Transporter Gene *mntC* Attenuate *Neisseria gonorrhoeae* In Vivo but Not by Increasing Susceptibility to Oxidative Killing by Phagocytes[▽]

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The hallmark of gonorrhea is an intense inflammatory response that is characterized by polymorphonuclear leukocytes (PMNs) with intracellular gonococci. A redundancy of defenses may protect *Neisseria gonorrhoeae* from phagocyte-derived reactive oxygen species. Here we showed that a gonococcal catalase (*kat*) mutant in strain MS11 was more sensitive to H_2O_2 than mutants in cytochrome *c* peroxidase (*ccp*), methionine sulfoxide reductase (*msrA*), or the metal-binding protein (*mntC*) of the MntABC transporter. *kat ccp* and *kat ccp mntC* mutants were significantly more sensitive to H_2O_2 than mutants in any single factor. None of the mutants showed increased susceptibility to murine PMNs. Recovery of the *mntC* and *kat ccp mntC* mutants from the lower genital tract of BALB/c mice, but not the *kat* or *kat ccp* mutants, was significantly reduced relative to wild-type bacteria. Interestingly, unlike the MS11 *kat* mutant, a *kat* mutant of strain FA1090 was attenuated during competitive infection with wild-type FA1090 bacteria. The FA1090 *kat* mutant and MS11 *mntC* mutant were also attenuated in mice that are unable to generate a phagocytic respiratory burst. We conclude that inactivation of three well-characterized antioxidant genes (*kat*, *ccp*, and *mntC*) does not increase gonococcal susceptibility to the phagocytic respiratory burst during infection and that gonococcal catalase and the MntC protein confer an unidentified advantage in vivo. In the case of catalase, this advantage is strain specific. Finally, we also showed that an *msrA* mutant of strain MS11 demonstrated delayed attenuation in BALB/c but not C57BL/6 mice. Therefore, MsrA/B also appears to play a role in infection that is dependent on host genetic background.

Neisseria gonorrhoeae is a facultative anaerobic bacterium with no environmental or animal reservoir outside of humans. *N. gonorrhoeae* is maintained in the human population on mucosal surfaces, including the urethra, cervix, pharynx, and rectum. Tissues of the upper reproductive tract of both males and females can also be infected. As is typical of the pyogenic cocci, *N. gonorrhoeae* induces an intense inflammatory response during symptomatic infections that is characterized by numerous polymorphonuclear leukocytes (PMNs) with intracellular gram-negative diplococci (21).

The mechanism(s) by which the gonococcus evades oxidative killing by phagocytes is of particular interest based on evidence that a proportion of intracellular gonococci survives and even multiplies within the PMNs (5, 6, 34, 42, 54, 55) despite the induction of an intracellular respiratory burst (42, 58). Several factors protect *N. gonorrhoeae* from exposure to oxidative stress in vitro (reviewed in reference 43). A nonenzymatic quenching mechanism that is based on the accumulation of intracellular manganese through the MntABC transporter protects *N. gonorrhoeae* from H_2O_2 and superoxide anion (52), and high levels of catalase protect gonococci from H_2O_2 and exposure to paraquat (20, 25, 47). Cytochrome *c* peroxidase (Ccp) also increases gonococcal resistance to H_2O_2 (53), and methionine sulfoxide reductase (MsrA/B), which repairs methionine sulfoxide residues on oxidatively damaged proteins, confers increased resistance to H_2O_2 and extracellularly generated reactive oxygen species (ROS) (45). Several other gonococcal factors that detoxify H_2O_2 and/or ROS have been identified (8, 41, 50, 59).

Many physiological factors can affect interactions between *N. gonorrhoeae* and PMNs. These factors include iron concentration, O_2 tension, pH (17), lactate (3), and the presence of CMP neuraminic acid (18, 38, 58). Environmental factors also regulate the expression of genes that protect *N. gonorrhoeae* from H_2O_2 and ROS in vitro (43). The balance of these physiological factors is difficult to reproduce in vitro. Infection of estradiol-treated BALB/c mice with *N. gonorrhoeae* causes a localized inflammatory response as evidenced by elevated numbers of PMNs and macrophages in vaginal and cervical tissue from infected mice (48). We recently reported that a catalase (*kat*) mutant of *N. gonorrhoeae* strain FA1090 can establish experimental murine infection despite the induction of a vigorous PMN response. High numbers of catalase-deficient gonococci were seen within PMNs and there was no significant difference in the number of wild-type or *kat* mutant gonococci recovered (46). That report was the first demonstration that catalase-deficient gonococci can persist during periods of inflammation in an in vivo system. We did not use the sensitive method of competitive infection to assess colonization of the *kat* mutant, however, or test the possibility that

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TABLE 1. *N. gonorrhoeae* strains used in this study

Strain	Description	Reference
MS11	Wild type, PorB.1B, Sm^r	51
FA1090	Wild type, PorB.1B, Sm^r	9
GP301	*kat*::Em^r (strain MS11)	This study
GP302	*ccp*::Cm^r (strain MS11)	This study
GP303	*msrA*::Cm^r (strain MS11)	This study
GP304	*mntC*::Km^r (strain MS11)	This study
GP311	*kat*::Em^r *ccp*::Cm^r (strain MS11)	This study
GP314	*kat*::Em^r *msrA*::Cm^r (strain MS11)	This study
GP315	*msrA*::Cm^r, *ccp*::Km^r (strain MS11)	This study
GP316	*mntC*::Km^r, *kat*::Em^r (strain MS11)	This study
GP317	*mntC*::Km^r *ccp*::Cm^r (strain MS11)	This study
GP318	*mntC*::Km^r *kat*::Em^r *ccp*::Cm^r (strain MS11)	This study
GP500	$kat_{\Delta 47-1384}$::*aphA-3* (strain FA1090)	47
GP506	GP500 complemented with kat_{MS11}	This study

TABLE 2. Nucleotide primers used in this study

Primer	Sequence (5′–3′)[a]
Kat2R[b]	.....TGAGTACTGACCAACCTGAGAAAAGGA
Kat2F[b]	.....TGAGTACTAAAAAACGCCCACTGAAAC
pG3EmR	.....TGACTGCGCTTACCCTTCCT
pG3EmF	.....AGGCAGGAGAGGGATTCGTC
ccpR[c]	.....ACGCGTCGACCTCCCATAAGGAATACACG
ccpF[c]	.....ACGCGTCGACAGCCGATGGTAATGGTAGC
mntR[c]	.....ACGCGTCGACACAGGAAAACAGTTATGAAAC
mntF[c]	.....ACGCGTCGACTGAATGTAAACCGGTGAGG
msrR[c]	.....ACGCGTCGACCGTCCACACAGGAAACATC
msrF[c]	.....ACGCGTCGACGCGATGCTGTCTGAACAAATC

[a] Enzyme restriction sites (underlined) were included in these primers for cloning purposes.
[b] Primer contains a ScaI restriction site.
[c] Primer contains a SalII restriction site.

functionally redundant factors may mask any attenuation due to the absence of catalase.

Here we examined the contribution of four well-characterized antioxidant factors toward *N. gonorrhoeae* resistance to oxidative killing by phagocytes in the mouse infection model. To this end, we constructed single, double, and triple mutants in the *kat*, *ccp*, *msrA*, and/or *mntC* genes in the same strain background. Mutants that showed the most sensitivity to H_2O_2 in vitro were tested for the capacity to colonize BALB/c mice during competitive infection with the wild-type parent strain. Mutants that were attenuated in BALB/c mice were tested in mice that are deficient in the Phox91 subunit of the NADPH oxidase complex to determine if evasion of phagocytic respiratory burst was the basis of the attenuation.

MATERIALS AND METHODS

Bacterial strains. A description of the bacteria used in this study is provided in Table 1. *N. gonorrhoeae* strains MS11 and FA1090 have been extensively characterized in the male volunteer model of experimental urethritis (9, 40, 51). The *kat*, *ccp*, *msrA*, and *mntC* genes of strain MS11 were isolated by PCR amplification of MS11 chromosomal DNA using primers Kat2R and Kat2F (for *kat*), ccpR and ccpF (for *ccp*), msrR and msrF (for *msrA*), and mntR and mntF (for *mntC*) (Table 2). The primers were designed to allow amplification of the 10-bp neisserial DNA uptake sequence (13) found downstream of each gene. PCR primers were designed based on information from the *N. gonorrhoeae* FA1090 genome sequence database (http://www.genome.ou.edu/gono.html). The resultant PCR products (1,697 bp [*kat*], 1,464 bp [*ccp*], 1,337 bp [*mntC*], and 1,708 bp [*msrA*]) were ligated to the PCR Blunt vector (Invitrogen) and electroporated into *Escherichia coli* Top10. Km^r transformants were screened for the desired clones and confirmed by nucleotide sequence analysis. Cloned genes were digested with a restriction enzyme that cut within a unique site in each open reading frame (NdeI for *kat*, HindIII for *ccp*, BsgI for *mntC*, and SspI for *msrA*). The linearized plasmids were ligated with a chloramphenicol acetyltransferase (*cat*) gene contained on an AgeI and XbaI fragment from pGCC5 (for *ccp* and *msrA*), an erythromycin resistance gene (Em^r) amplified from pGCC3 with the primers pG3EmR and pG3EmF (for *kat*), or a nonpolar *aphA-3* kanamycin resistance (Km^r) cassette contained on an 840-bp SmaI fragment from pUC18Km (31) (for *mntC*). Plasmids with the insertionally inactivated genes were cut at the restriction site incorporated into the respective primers (Table 2) to generate linear fragments, which were then transformed into strain MS11. Transformants were selected on agar with the appropriate antibiotics to isolate single mutants GP301, GP302, GP303, and GP304 (Table 1). Double mutants GP311 and GP314 were generated by transforming linear plasmid DNA that contained insertionally inactivated *ccp* and *msrA* genes, respectively, into the *kat* mutant GP301. Double mutant GP315 was generated by introducing the *ccp*::Km^r gene into mutant GP303. Mutants with inactivated *mntC* genes and *kat* (GP316) or *ccp* (GP317) genes were isolated after transformation of linearized plasmids carrying the *kat*::Em^r or *ccp*::Cm^r genes into GP304 bacteria. The triple mutant GP318 (*mntC kat ccp*) was constructed by introducing a *ccp*::Cm^r gene into mutant GP316.

The *kat* mutant GP500 (*kat*::*aphA3*) is in *N. gonorrhoeae* strain FA1090 and was described previously (47). For complementation of GP500, a wild-type copy of the MS11 *kat* gene without its own promoter was cloned into the PCR Blunt vector using primers that produced ScaI sites as described previously (58). The resultant plasmid was digested with ScaI and the 1,697-kb fragment that contains the *kat* gene was cloned into the ScaI site of pGCC4 (30). An 8.6-kb fragment that contains the *kat* gene and sequences that correspond to a nonessential region between the *lctP* and *aspC* genes on the gonococcal chromosome was transformed into *kat* mutant GP500. Transformants were selected on GC agar with 1 μg/ml of Em, and a colony that exhibited catalase activity upon exposure to a few drops of H_2O_2 was passaged and frozen as strain GP506. The occurrence of the desired allelic exchange in GP506 bacteria was confirmed by PCR. All transformations were performed by the method of Gunn and Stein (19). Neisserial Insertional Complementation System vectors pGCC3, pGCC4, and pGCC5 (29, 30) were provided by H. S. Seifert, Northwestern University.

Culture conditions and growth curves. All *Neisseria* strains were cultured in 7% CO_2 at 37°C on GC agar or in GC broth (GCB) supplemented with Kellogg's supplement and 12 μM $Fe(NO_3)_3$ (22). Luria agar with Em (300 μg/ml), Km (50 μg/ml), Cm (50 μg/ml), tetracycline (50 μg/ml), or ampicillin (100 μg/ml [for Luria both] or 200 μg/ml [for Luria plates]) was used for plasmid maintenance or to isolate *E. coli* carrying recombinant plasmids. GC agar with Em (0.5 μg/ml [for FA1090] or 3 μg/ml [MS11]), Km (50 μg/ml), or Cm (10 μg/ml) was used to isolate mutants following allelic exchange. GC agar containing vancomycin, colistin, nystatin, trimethoprim, and streptomycin sulfate (GC-VNTS agar), GC agar with streptomycin (Sm; 100 μg/ml), or GC agar with Sm and Cm, Km, or Em at the above concentrations was used to culture vaginal mucus in mouse infection experiments. VCNT supplement and all media were from Difco. All other antibiotics were from Sigma. Growth kinetics of wild-type and mutant gonococci were determined by culturing bacteria in supplemented GCB with 5 mM $NaHCO_3$ at 37°C with aeration and measuring the change in absorbance at 600 nm (A_{600}) over time. For cocultures (in vitro competition assays), similar numbers of wild-type MS11 and mutant gonococci were inoculated into supplemented GCB, and aliquots were cultured on GC agar with and without the appropriate antibiotic selection at hourly time points through mid-stationary phase. The number of CFU on GC with Em (for GP301 or GP311), Km (for GP304, GP500, or GP506), or Cm (for GP303) was subtracted from the number of CFU on GC agar without antibiotics (total CFU) to determine the relative number of wild-type and mutant gonococci over time.

Catalase activity. Catalase activity was measured in whole-cell lysates of stationary-phase cultures grown in supplemented GCB as described previously (47) except that sonication was used to break bacterial cells (level 3, 15 s on and 30 s off, 3 min total) and bacteria were suspended in 50 mM potassium phosphate (monobasic) buffer before lysis. Catalase activity was expressed as units/mg of protein using the following formula: units/mg = $(\Delta A_{240}/\text{min} \times 1{,}000)/(43.6 \times \text{mg}$ of enzyme/ml of reaction mixture). Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM; Sigma) was used to induce transcription of the *kat* gene in strain GP506.

Sensitivity to H_2O_2 and inducers of ROS. Wild-type and mutant gonococci were cultured aerobically in a 5% CO_2 incubator or anaerobically overnight in an anaerobic jar in the presence of 2 mM nitrite (27). H_2O_2 and paraquat sensitivities were determined via a disc diffusion assay (47). Zones of growth inhibition were calculated by determining the diameter (in mm) of the region in which no

bacteria grew minus the diameter of the disc. All experiments were performed at least twice to test reproducibility.

Experimental murine infection. Six- to 8-week-old female BALB/c mice (National Cancer Institute), C57BL/6J mice, and B6.129S6-Cybb^{tm1Din}/J mice, which is a Phox-deficient mouse line in the C57BL/6J background (Jackson Laboratories), were treated with 17β-estradiol and antibiotics to promote long-term colonization by *N. gonorrhoeae* as described previously (23, 24). For noncompetitive infections, 10^6 CFU of wild-type MS11 or GP318 (*kat ccp mntC*) gonococci were inoculated intravaginally into separate groups of mice ($n = 8$ mice/group), and vaginal mucus was quantitatively cultured every other day on GC-VCNTS agar. For competitive infections, groups of five to eight mice were inoculated with a mixed suspension that contained similar numbers of wild-type and mutant bacteria, and the relative recovery of the mutant over time was determined using the appropriate selective agar as described previously (58). Competitive indices (CI) were calculated as the ratio of mutant to wild-type bacteria recovered (output) divided by the ratio of mutant to wild-type bacteria in the inoculum (input). All animal experiments were conducted in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol approved by the University's Institutional Animal Care and Use Committee.

PMN assays. Murine PMNs were elicited by peritoneal lavage, and opsonophagocytic killing of *N. gonorrhoeae* was performed via a tumbling tube assay as described previously (16, 58). Results were expressed as percent survival {100 × [(number of CFU recovered at 90 min)/(number of CFU recovered at time zero)]} (4). Induction of the phagocytic respiratory burst in PMNs incubated with wild-type or mutant gonococci was measured via a luminol- and isoluminol-enhanced chemiluminescence (CL) assay (4, 58). Experiments were performed on three separate occasions with similar results. The Phox-deficient and Phox-sufficient phenotypes of B6.129S6-Cybb^{tm1Din}/J and C57BL/6J mice were confirmed by measuring the CL response of PMNs from these mice upon stimulation with 10 ng/ml phorbol myristate acetate and by nitroblue tetrazolium staining (36).

Quantitative reverse transcription-PCR (RT-PCR). A modification of the conditions used by Packiam et al. (33) was used to measure in vivo expression of the wild-type *kat* gene in strain FA1090 and a recombinant *kat* gene in the complemented mutant GP506 in vivo. Mice were infected with wild-type, GP500 *kat* mutant, or GP506-complemented mutant bacteria that had been cultured in the presence or absence of 0.1 mM IPTG. Total RNA was extracted from wild-type inoculum suspensions and from vaginal swab suspensions from infected and uninfected mice using the Qiagen mini RNAeasy isolation kit. All preparations were treated twice with DNase I (6 U, RNase free; Ambion) and stored at −70°C until use. cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) per the manufacturer's instructions. Control samples containing nuclease-free water instead of reverse transcriptase were tested in parallel to rule out contaminating DNA as the source of any PCR product. The SYBR Green Master Mix kit (ABI) was used to perform real-time PCR assays. cDNA reaction mixtures (20 μl) were diluted to a final volume of 100 μl with nuclease-free double-distilled water. Two microliters of the diluted cDNA template and 1:2 and 1:4 dilutions thereof were subjected to PCR amplification in the Applied Biosystems 7500 Real-Time PCR system in a total volume of 25 μl containing 12.5 μl SYBR Green Master Mix, 1 μl of each primer (0.4 μM, final concentration), and 8.5 μl double-distilled H_2O. Reaction conditions were 10 min at 95°C, 40 15-s cycles of 95°C, and 1 min at 60°C. Data were analyzed using the Sequence Detector v.1.7a software (ABI). The cycle threshold (C_T) was defined as the cycle number that corresponded to the point at which the amplification plot of the samples was linear. The comparative C_T method ($\Delta\Delta C_T$) was used to measure *kat* expression relative to that of *rmp*, which encodes the reduction-modifiable membrane protein as the active reference control (normalizer). Quantification of relative *kat* expression was based on the difference between the C_T values of the normalizer (*rmp*) and the C_T values of individual samples: $\Delta C_T = C_T(rmp) - C_T(\text{sample})$. The difference between the ΔC_T value of the wild-type or GP506 samples and the ΔC_T value of the wild-type inoculum ($\Delta\Delta C_T$) was used to obtain an absolute value for the difference in *kat* mRNA levels between samples ($2^{\Delta\Delta}C_T$). Results are expressed in arbitrary units to reflect this difference.

Data analysis. An unpaired *t* test was used to evaluate differences in susceptibility to H_2O_2, paraquat, and PMN killing and to compare the average duration of recovery for mice. All statistical analyses were performed with SPSS software (Chicago, IL).

RESULTS

In vitro analysis of mutant phenotypes. The sensitivities of MS11 mutants with an insertionally inactivated *kat*, *ccp*, *msrA*, or *mntC* gene and different combinations thereof to H_2O_2 or paraquat were measured to confirm the expected phenotypes and to determine if loss of more than one factor had an additive effect. We were particularly interested in the sensitivity of the mutants under anaerobic conditions, since the O_2 tension of the female genital tract is reduced (35, 56). Similar to findings reported by others (26, 45, 47, 52), the *kat*, *mntC*, and *msrA* single mutants were more sensitive to H_2O_2 than the wild-type strain under aerobic conditions ($P < 0.05$). Consistent with the fact that *ccp* is only expressed during O_2-limited growth (53), the *ccp* mutant was not more sensitive under aerobic conditions (data not shown). When cultured anaerobically, the *kat, ccp*, *msrA*, and *mntC* mutants showed a dose-dependent H_2O_2 sensitivity that was greater than that of the wild-type strain, although the H_2O_2 sensitivity phenotype of the *msrA* mutant was not consistently different over all doses tested (Fig. 1A). Compared to the other single mutants, the *kat* mutant GP301 was more sensitive to H_2O_2 at a statistically higher level ($P < 0.001$).

Inactivation of both the *kat* and *ccp* genes (mutant GP311), but not the *kat* and *msrA* genes (mutant GP314) or *kat* and *mntC* genes (mutant GP316) (Fig. 1A), caused a higher level of H_2O_2 sensitivity than that exhibited by the single *kat* mutant GP301 when examined at the highest concentration tested (80 mM H_2O_2) ($P < 0.05$). The H_2O_2 sensitivity of mutant GP318, which carries three inactivated genes, *kat*, *ccp*, and *mntC*, was similar to that of the *kat ccp* double mutant (Fig. 1A). Inactivation of the *ccp* gene increased the H_2O_2 sensitivity of the *mntC* mutant to the level conferred by inactivation of *ccp* alone when tested at 80 mM H_2O_2 ($P < 0.007$). Inactivation of *ccp* along with *msrA* (mutant GP315) did not have an additive effect over mutation of either single gene. We concluded that when gonococci are cultured anaerobically, catalase is the most important factor for protecting against H_2O_2 and that mutation of *ccp* but not *mntC* or *msrA* increases the H_2O_2 sensitivity of the *kat* mutant. Additionally, loss of *ccp* can elevate the H_2O_2 sensitivity of an *mntC* mutant, but not an *msrA* mutant, when cultured anaerobically.

Paraquat, which induces the production of intracellular ROS, was more toxic to the *kat* mutant GP301 and *mntC* mutant GP304 than wild-type MS11 bacteria under aerobic conditions, as reported for mutants in these factors in other strains (47, 52) (data not shown). When tested anaerobically, the *mntC*, *ccp*, and *kat* single mutants, the *mntC kat*, *mntC ccp*, and *kat ccp* double mutants, and the *mntC ccp kat* triple mutant were equally more sensitive to paraquat than the wild-type strain ($P < 0.05$) (Fig. 1B). We conclude that inactivation of *kat* or *mntC* causes strain MS11 to be less able to tolerate intracellular ROS under anaerobic conditions. However, in contrast to sensitivity to exogenously added H_2O_2, mutation of these factors or of *msrA* along with *kat* or *mntC* does not have an additive effect.

Assessment of in vivo fitness. We next tested the capacity of mutants with the highest degree of H_2O_2 sensitivity in vitro to establish genital tract infection in female mice. Based on the likelihood that inactivation of the *mntC*, *ccp*, and *kat* genes

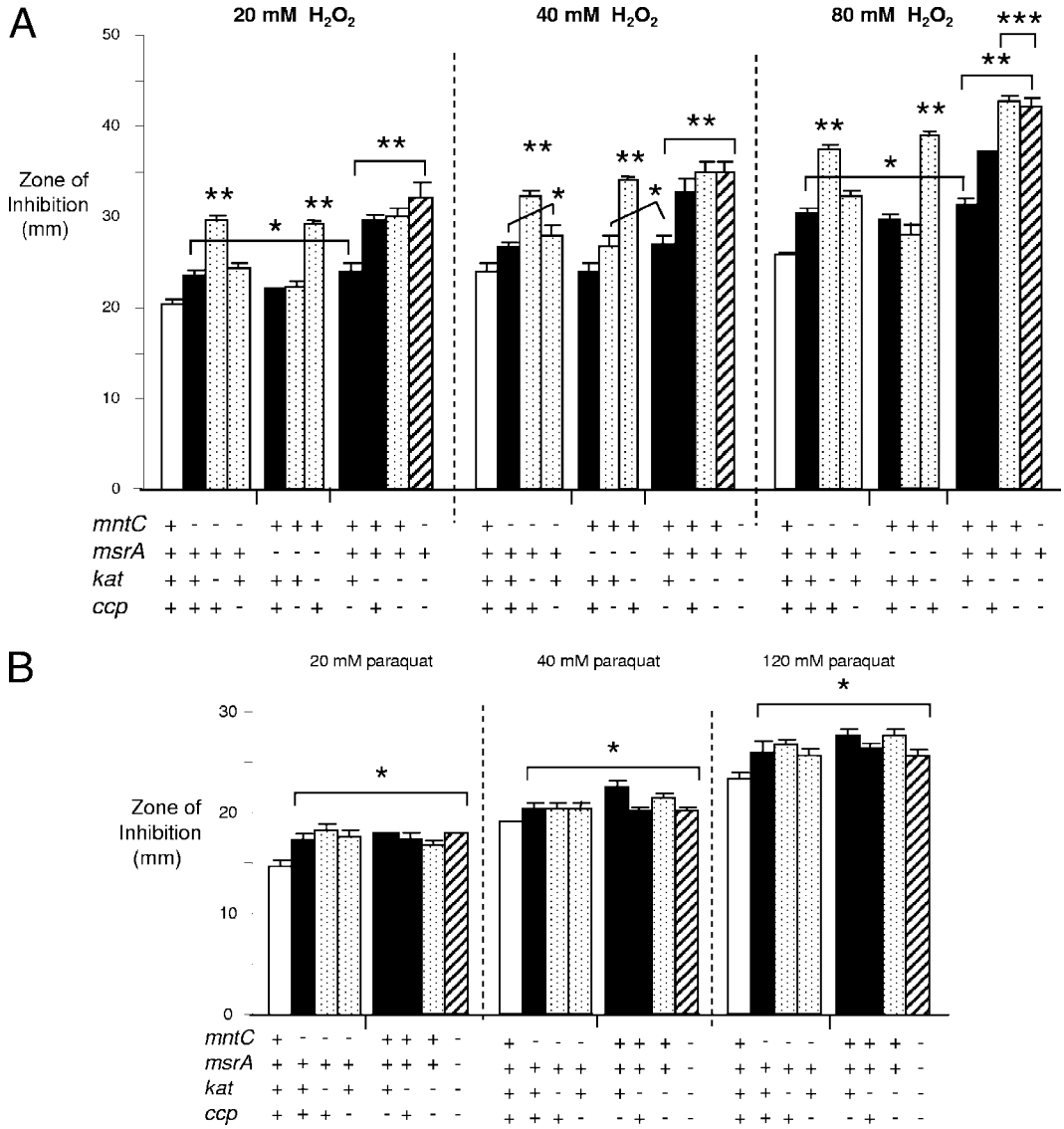

FIG. 1. Sensitivity of antioxidant mutants to H_2O_2 and paraquat. Wild-type MS11 and mutant gonococci were tested for sensitivity to increasing concentrations of H_2O_2 (A) or paraquat (B) in a disc diffusion assay under anaerobic conditions in the presence of 2 mM nitrite. Results for the wild-type strain (open bars), single mutants (black bars), double mutants (stippled bars), and the triple mutant GP318 (striped bar) are shown and represent the average zone of inhibition and standard deviation as calculated from the results of triplicate assays. The experiment was repeated once to test reproducibility and the results were similar. *, $P < 0.05$ (compared to wild-type strain); **, $P < 0.001$ (compared to wild-type strain); ***, $P < 0.05$ (compared to *kat* mutant GP301).

would have the most profound effect in vivo, we inoculated groups of mice with 10^6 CFU of wild-type MS11 bacteria or the *kat ccp mntC* mutant GP318 and measured the number of gonococci recovered over 12 days. We found no difference in the duration of infection, with mice colonized with MS11 or GP318 bacteria for an average of 8.3 or 7.5 days, respectively (range, 2 to 12 days [wild type] or 4 to 12 days [mutant]). There was also no difference in the number of bacteria recovered over time (Fig. 2A). We therefore next tested the capacity of the *mntC kat ccp* mutant to compete with the wild-type strain in vivo, which is a more sensitive technique than noncompetitive infection. Groups of mice were inoculated with mixed suspensions containing similar numbers of GP318 and MS11 bacteria, and the ratio of mutant to wild-type bacteria among vaginal isolates was compared to that of the inoculum. Significantly reduced recovery of GP318 bacteria occurred relative to the wild-type strain over time, with a 10-fold decrease in the mean CI detected 2 days after inoculation. By day 8, a 1,000-fold decrease in the mean CI was detected, and high numbers of wild-type bacteria but no GP318 bacteria were recovered from a majority of mice on days 8 and 10 postinoculation (Fig. 2B). No differences in the growth rate (Fig. 2C) or ratio of mutant to wild-type gonococci (Fig. 2D) were observed when wild-type and GP318 mutant bacteria were cultured together under aerobic conditions.

To determine which gene(s) contributed to the attenuated phenotype of mutant GP318, competitive infections were performed between wild-type MS11 bacteria and the *kat* mutant

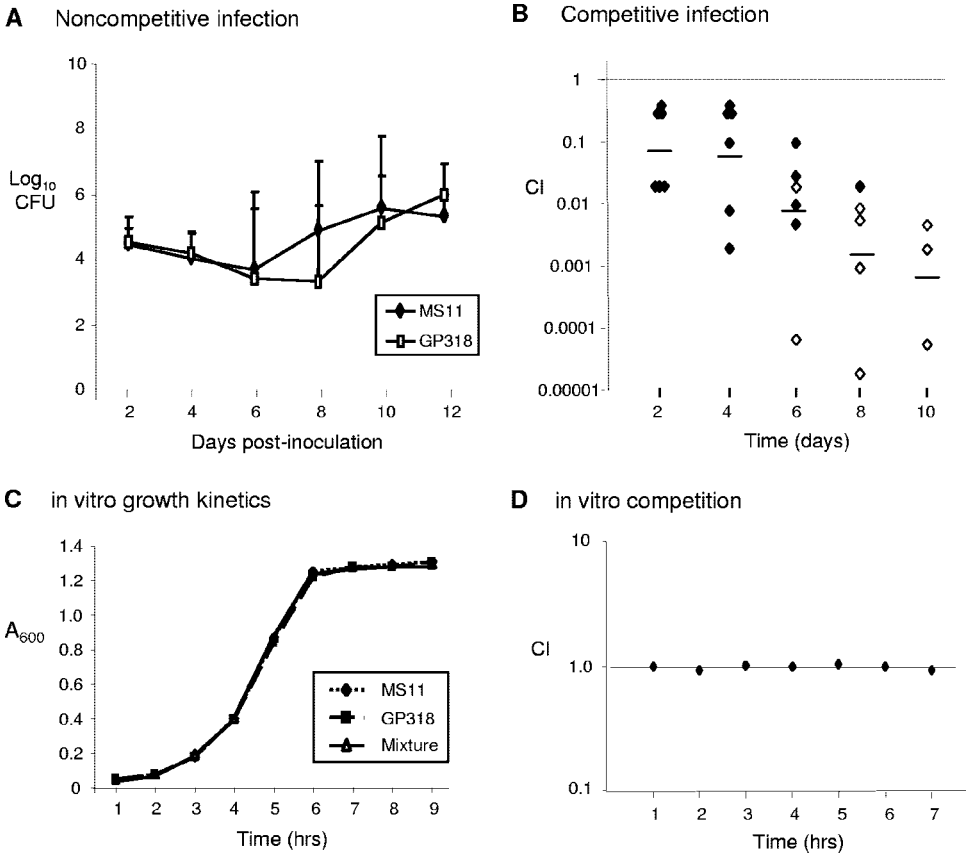

FIG. 2. A *kat ccp mntC* mutant is attenuated during competitive infection with the wild-type strain. The fitness of the triple *kat ccp mntC* mutant GP318 was compared to that of the wild-type strain in noncompetitive and competitive infections of estradiol-treated BALB/c mice. (A) For noncompetitive infections, groups of mice ($n = 8$) were inoculated with wild-type MS11 or mutant GP318 bacteria, and vaginal swab suspensions were quantitatively cultured over time. There was no difference in the number of wild-type or mutant gonococci recovered from either group. (B) For competitive infections, a mixed suspension containing similar numbers of wild-type MS11 and mutant GP318 gonococci were inoculated into mice. The relative recovery of the mutant over time was determined using GC agar with Sm (total number) and GC agar with Sm and Cm (mutant) as described in Materials and Methods. Results shown are the CI for individual mice at each time point. A CI of <1.0 indicates a decrease in the ratio of mutant to wild-type gonococci compared to that of the inoculum and thus decreased fitness. Horizontal bars represent the geometric mean. Open symbols represent mice from which no mutants were recovered; the limit of detection (1 CFU per 100 μl of swab suspension) was used as the number of mutant CFU recovered in these cases. (C) The growth kinetics of wild-type MS11 and mutant GP318 when incubated separately in GC broth under aerobic conditions or together was measured by the change in the A_{600} over time. (D) Ratio of mutant to wild-type gonococci recovered from the mixed broth culture shown in panel C divided by the ratio at time zero. The values shown (in vitro CI) are approximately 1.0 at each time point tested, which indicates the mutant has no advantage or disadvantage compared to the wild-type strain when cultured under these conditions in vitro.

GP301, the *kat ccp* double mutant GP311, and the *mntC* mutant GP304. Despite the clear advantage that is afforded by catalase when gonococci are exposed to H_2O_2 in vitro, the absence of catalase alone or of both catalase and Ccp did not cause attenuation in vivo, as evidenced by an average CI of 1.0 over the 10-day experiment (Fig. 3A and B). Em^r vaginal isolates (GP301 and GP311) on the primary isolation plates were flooded with H_2O_2, and no bubbles were observed; therefore, the *kat* mutation did not revert in vivo. We also detected expression of *ccp* in vaginal swab suspensions from mice infected with wild-type gonococci by RT-PCR (data not shown). This result is consistent with the O_2-limited environment of the mouse vagina and rules out the possibility that lack of expression of the wild-type gene during competitive infections with the *kat ccp* mutant was responsible for our not seeing a difference in the survival of Ccp-deficient gonococci in the mouse model.

In contrast to the *kat* and *kat ccp* mutants, the *mntC* mutant GP304 was attenuated relative to the wild-type strain. The relative recovery of GP304 bacteria decreased over time, with a 60- to 100-fold decrease in the mean CI detected by days 4 to 10 postinoculation (Fig. 3C). Repeat experiments showed similar results in terms of the degree of attenuation of the *mntC* mutant. Mutants GP301, GP311, and GP304 showed no growth advantage or disadvantage compared to the parent strain MS11 when cultured in GCB in vitro (data not shown). These results are consistent with inactivation of *mntC* being responsible for the attenuated phenotype of the *mntC kat ccp* triple mutant in vivo.

Strain-specific differences in the catalase mutants. We previously reported that a *kat* mutant in strain FA1090 colonized female BALB/c mice and persisted during a vigorous PMN influx. A nonsignificant, but dose-dependent, trend toward reduced duration of infection by the *kat* mutant was observed

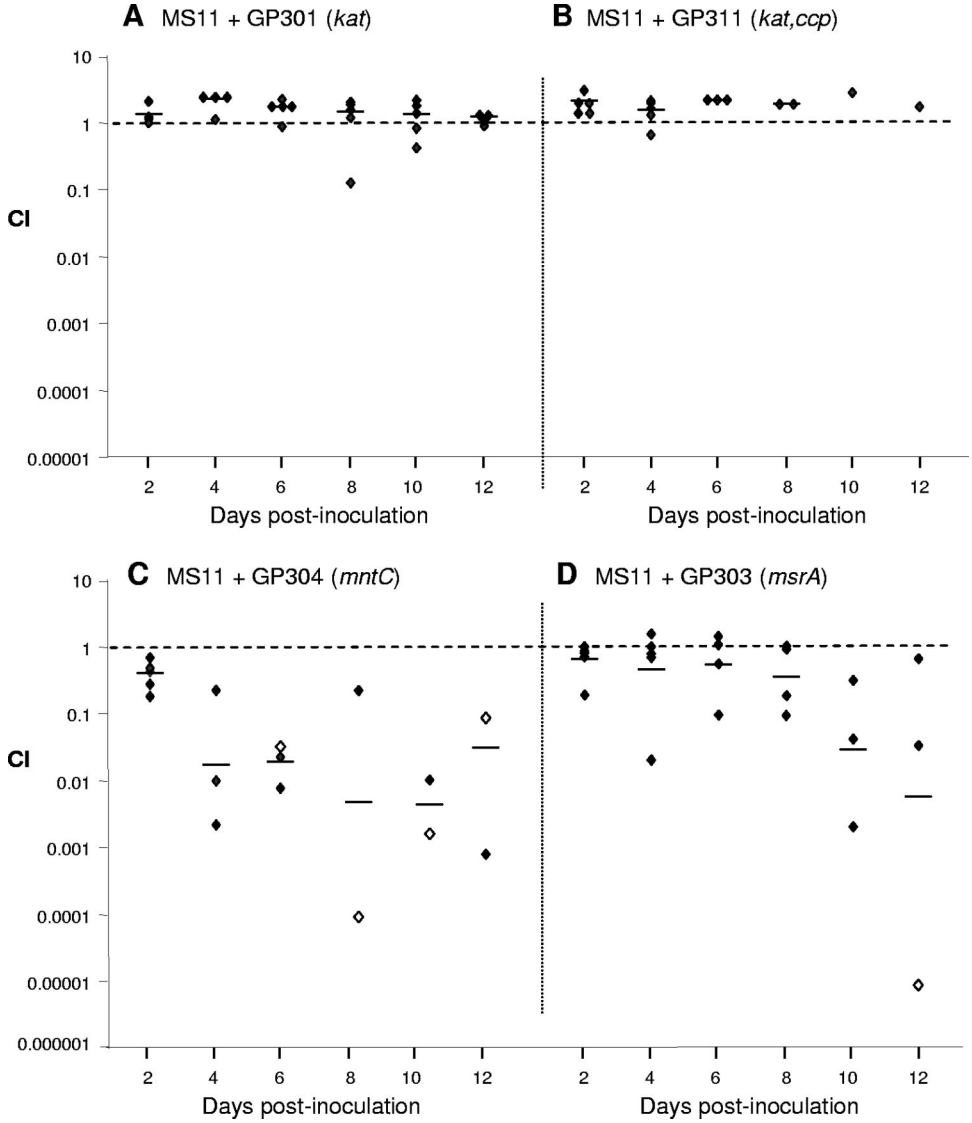

FIG. 3. Inactivation of *mntC* or *msrA* but not *kat* or *kat* and *ccp* in strain MS11 causes attenuation in BALB/c mice. Female estradiol-treated BALB/c mice were inoculated with similar numbers of wild-type MS11 gonococci mixed with GP301(*kat*) (A), GP311 (*kat ccp*) (B), GP304 (*mntC*) (C), or GP303 (*msrA*) (D) bacteria. The relative recovery of each mutant over time was determined using GC agar with Sm (total number) and GC agar with Sm plus Em (GP301), Cm (GP311 or GP303), or Km (GP304) as described in Materials and Methods. Results shown are the CI for individual mice at each time point. Horizontal bars represent the geometric mean. A CI of <1.0 indicates a decrease in the ratio of mutant to wild-type gonococci compared to that of the inoculum and thus decreased fitness. Open symbols represent mice from which no mutants were recovered; the limit of detection (1 CFU per 100 μl of swab suspension) was used as the number of mutant CFU recovered in these cases. Mutants GP303 and GP304 were tested twice in competitive infections with the wild-type strain (n = 5 to 8 mice per experiment), and the results were reproducible.

when compared to wild-type gonococci inoculated into separate groups of mice (46). Therefore, here we utilized the more sensitive technique of competitive infection to compare the fitness of GP500 bacteria relative to wild-type FA1090 bacteria in vivo. Interestingly, and in contrast to the MS11 *kat* mutant, the FA1090 *kat* mutant GP500 was dramatically attenuated relative to strain FA1090, with a 100- to 10,000-fold decrease in CI on days 2 to 6 postinoculation. No *kat* mutant bacteria were recovered from five of six mice by day 6 of infection, in contrast to high numbers of wild-type gonococci (10^3 to $>10^5$ CFU/100 μl of vaginal swab suspension) recovered at this time point (Fig. 4A).

Previous attempts to fully complement mutant GP500 were unsuccessful due to unstable expression (46) or production of an aberrant catalase protein by the recombinant *kat* gene (47). Here, we successfully complemented mutant GP500 by integrating a wild-type copy of the MS11 *kat* gene into a noncoding region of the chromosome as described in Materials and Methods. Complemented strain GP506 produced bubbles upon exposure to H_2O_2 and wild-type levels of catalase (Table 3) when cultured in the presence of IPTG. The H_2O_2 resistance of GP506 bacteria was also similar to that of the wild-type strain, and strain GP506 produced a catalase protein with the same migration as the wild-type species on activity gels (data not

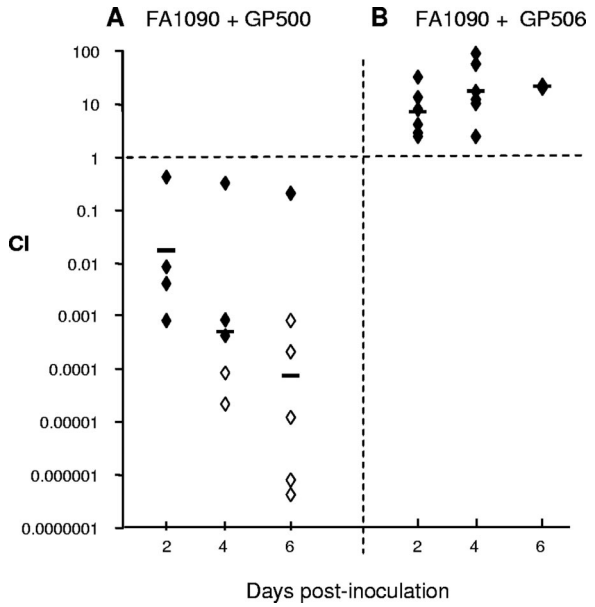

FIG. 4. An FA1090 *kat* mutant is attenuated during competitive infection with the wild-type strain. Female estradiol-treated BALB/c mice were inoculated with similar numbers of wild-type FA1090 bacteria mixed with GP500 (*kat*) or GP506 (complemented mutant) gonococci. The relative recovery of each strain was determined over time by culture on GC media with Sm (total number) or GC with Sm and Km (GP500 and GP506) as described in Materials and Methods. Results shown are the CI for individual mice at each time point. Horizontal bars represent the geometric mean. A CI of <1.0 indicates a decrease in the ratio of mutant to wild-type gonococci compared to that of the inoculum and thus decreased fitness. CI values of >1.0 indicate increased fitness. Open symbols represent mice from which no mutants were recovered; the limit of detection (1 CFU per 100 μl of swab suspension) was used as the number of mutant CFU recovered in these cases. The experiment was repeated and gave reproducible results. No fitness difference was observed when wild-type and *kat* mutant bacteria were cocultured in vitro.

shown). When tested in competitive infections with the parent strain FA1090, GP506 bacteria showed no evidence of attenuation, and in fact, the complemented mutant demonstrated a fitness advantage relative to the wild-type strain as indicated by a 10-fold increase in the mean CI within 2 days after inoculation (Fig. 4B).

The *kat* gene used to complement strain GP506 lacks its native promoter region and is under the control of the *lac* promoter present in the complementation vector (30, 44). The GP506 bacteria used to inoculate mice were cultured on medium with IPTG, but we did not administer IPTG to the mice to maintain expression of the *kat* gene over the course of infection. Therefore, to confirm that expression of the *kat* gene in strain GP506 occurred in vivo, quantitative RT-PCR was performed on vaginal washes from mice inoculated with wild-type, GP500, or GP506 bacteria cultured with or without IPTG. No PCR product was amplified from vaginal washes from mice infected with the *kat* mutant GP500 when *kat*-specific primers were used. Wild-type bacteria expressed the *kat* gene in vivo at a 10-fold-higher level than that expressed by in vitro-grown bacteria (Fig. 5, left panel). These results suggest *kat* expression is upregulated in the murine genital tract. In vivo expression of the *kat* gene in the complemented mutant was similar to wild-type expression levels when preinduced cultures were used to inoculate the mice (Fig. 5, center panel). A lower level of *kat* transcription was detected in mice inoculated with uninduced GP506 bacteria; however, expression was 5- to 33-fold higher than that of the *kat* gene expressed by in vitro-grown wild-type FA1090 bacteria (Fig. 5, right panel). We also observed variability in *kat* expression among mice inoculated with strain GP506 regardless of IPTG induction, with some mice expressing almost 1,000-fold more *kat* than that expressed by in vitro-grown wild-type bacteria. It is therefore possible that the observed in vivo fitness advantage of strain GP506 relative to the parent strain may be in part due to increased transcription of the recombinant *kat* gene when in the murine lower genital tract. The identity of the inducing factor is not known. Although *N. gonorrhoeae* does not produce a lactose permease, it is possible that vaginal lactose may induce expression from the *lac* promoter used in the complementation vector. However, we found no difference in the level of catalase produced by strain GP506 when cultured on media with no lactose and with 0.05 mM to 80 mM lactose (data not shown), and thus we ruled out this hypothesis. We conclude that an IPTG-like substance must be present in the murine genital tract that induces expression of the complementing *kat* gene in strain GP506 during infection.

Susceptibility to killing by PMNs from BALB/c mice. To determine whether any of the mutants were more susceptible to PMN killing in vitro, we compared the susceptibility of MS11 wild-type and mutant gonococci to PMNs isolated from BALB/c mice. All the MS11 mutants, including the *kat ccp mntC* mutant GP318, induced a CL response that was primarily intracellular, and there was no difference in the magnitude or kinetics of the CL response following exposure of PMNs to wild-type or mutant gonococci (Fig. 6A). As reported previously (58), wild-type MS11 gonococci were significantly killed by murine PMNs when opsonized with normal mouse serum and killing was abrogated by using serum heated to 56°C for 30 min. None of the MS11 mutants, including the *mntC kat ccp* triple mutant, showed increased susceptibility to PMN killing in vitro when cultured aerobically or under anaerobic conditions prior to the assay to express the *ccp* gene (data not shown). The CL data rule out the possibility that differences in the mutants' capacities to stimulate the phagocytic respiratory burst might influence the results. We conclude that none of the four factors tested, alone or in combination with one or two other factors, confers detectable resistance to PMN killing in vitro, including MntC, the loss of which attenuated strain MS11 in vivo.

Investigation of attenuated phenotypes in Phox-deficient mice. To further test the basis for the attenuation of the MS11

TABLE 3. Catalase expression in mutant and complemented strains

Strain	Catalase activity (units/mg) ± SD
FA1090	556 ± 32
GP500	0
GP506 with IPTG	628 ± 6
GP506 without IPTG	13 ± 4
MS11	567 ± 27
GP301	0

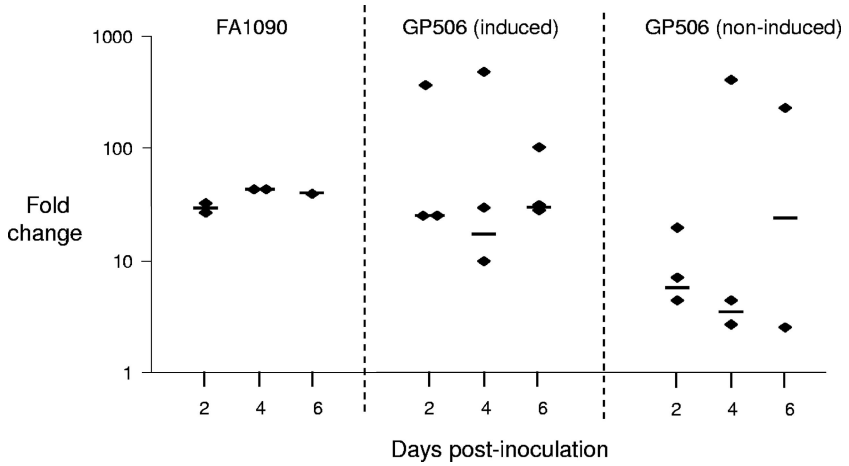

FIG. 5. In vivo expression of the *kat* gene in wild-type bacteria and the complemented *kat* mutant. Mice were inoculated with wild-type FA1090 (left panel), the complemented mutant GP506 cultured in IPTG (center panel), or GP506 bacteria cultured without IPTG (right panel). Vaginal washes from individual mice in each group were collected on days 2, 4, and 6 postinoculation, and RT-PCR was performed to measure expression of the wild-type or recombinant *kat* genes. Expression of *rmp* served as the active reference control (normalizer). Results are expressed as the fold difference compared to expression of the native *kat* gene in the wild-type FA1090 inoculum. The geometric mean is represented by the horizontal bar. No *kat* transcript was detected in vaginal washes from mice infected with the *kat* mutant GP500. Samples to which no reverse transcriptase was added were tested in parallel to control for contaminating DNA, and no PCR products were amplified from these samples.

mntC (GP304) and FA1090 *kat* (GP500) mutants we performed competitive infections in mice that carry a mutation in the 91-kDa subunit of the NADPH oxidase complex (Phox-deficient) and C57BL/6 (wild-type, Phox-sufficient) mice. We predicted that if increased susceptibility to phagocyte-derived ROS produced were responsible for the reduced recovery of these mutants, the mutants would not show an attenuated phenotype in Phox-deficient mice. We found that the recoveries of both GP304 and GP500 bacteria were markedly reduced in Phox-deficient mice relative to their respective wild-type parent strains, and there was no difference in the mean CI when results from the Phox-deficient and wild-type C57BL/6 backgrounds were compared (Fig. 7). We also tested the susceptibility of wild-type MS11 and the *mntC kat ccp* triple mutant GP318 to PMNs from Phox-deficient and normal C57BL/6 mice. Both wild-type MS11 and GP318 bacteria were killed by PMNs from either mouse strain, and there was no difference in the degree of killing with respect to the strain of mice from which the PMNs were isolated (Fig. 6B). We conclude that the MS11 *mntC* and FA1090 *kat* mutants are attenuated in vivo for reasons that are unrelated to increased susceptibility to the phagocytic respiratory burst.

Delayed attenuation of an *msrA* mutant in BALB/c mice. Inactivation of the *msrA* gene in strain MS11 singly or in combination with *ccp* or *kat* did not elevate H_2O_2 sensitivity to the level shown by the *kat* mutant alone at any concentration of H_2O_2 tested. Like mutants in *mntC*, *kat*, and *ccp*, the *msrA* mutant was not more sensitive to killing by isolated PMNs from BALB/c mice in vitro. We wished to test the *msrA* mutant in vivo, however, based on reports that *msr*A mutants in *Mycoplasma genitalium* (11) and *Helicobacter pylori* (1) were attenuated in animal infection models. Interestingly, *msrA* mutant GP303 showed significantly reduced recovery from BALB/c mice relative to wild-type MS11 bacteria as infection progressed beyond 8 days, with a mean 50-fold decrease in recovery on day 10 postinoculation (Fig. 3D). No MsrA/B-deficient bacteria were recovered from one mouse that was colonized with $>10^5$ CFU of wild-type gonococci on day 12 (Fig. 3D). We found no difference in the growth kinetics of wild-type and *msrA* mutant bacteria when cultured in broth through late stationary phase under aerobic conditions (data not shown), and the delayed attenuation phenotype was reproduced in a second competitive infection experiment. We next performed experiments with C57BL/6 mice and Phox-deficient mice to determine if the basis of the attenuation was related to the phagocytic respiratory burst. Surprisingly, mutant GP303 was not attenuated in the C57BL/6 background in each of two experiments (data not shown). The *msrA* mutant was equally susceptible to killing by PMNs from Phox-deficient mice compared to C57BL/6 mice (data not shown); however, without Phox-deficient mice in the BALB/c background, we were unable to more definitively test the role of NADPH oxidase in challenging the *msrA* mutant during infection.

DISCUSSION

The capacity of *N. gonorrhoeae* to persist in inflamed urogenital tissues is a remarkable feature of its pathogenesis. The mechanism by which *N. gonorrhoeae* evades oxidative killing by PMNs is not yet known, and a functional redundancy may mask the phenotype of mutants in one or a few of these factors. Others reported that a *kat* mutant and a *kat ccp* mutant of gonococcal strain 1291 were not more sensitive to killing by human PMNs (42). Here we constructed a series of mutants in strain MS11 that allowed us to test four well-characterized factors known to protect *N. gonorrhoeae* from oxidative stress in vitro. We found that catalase was clearly the most important factor in protecting against H_2O_2 and that only Ccp had an additive effect over catalase alone. Despite their in vitro phenotypes, the MS11 *kat* and *kat ccp* mutants showed no evidence of attenuation in a murine model of genital tract infection. In contrast, a *kat ccp mntC* mutant was attenuated in vivo, as was

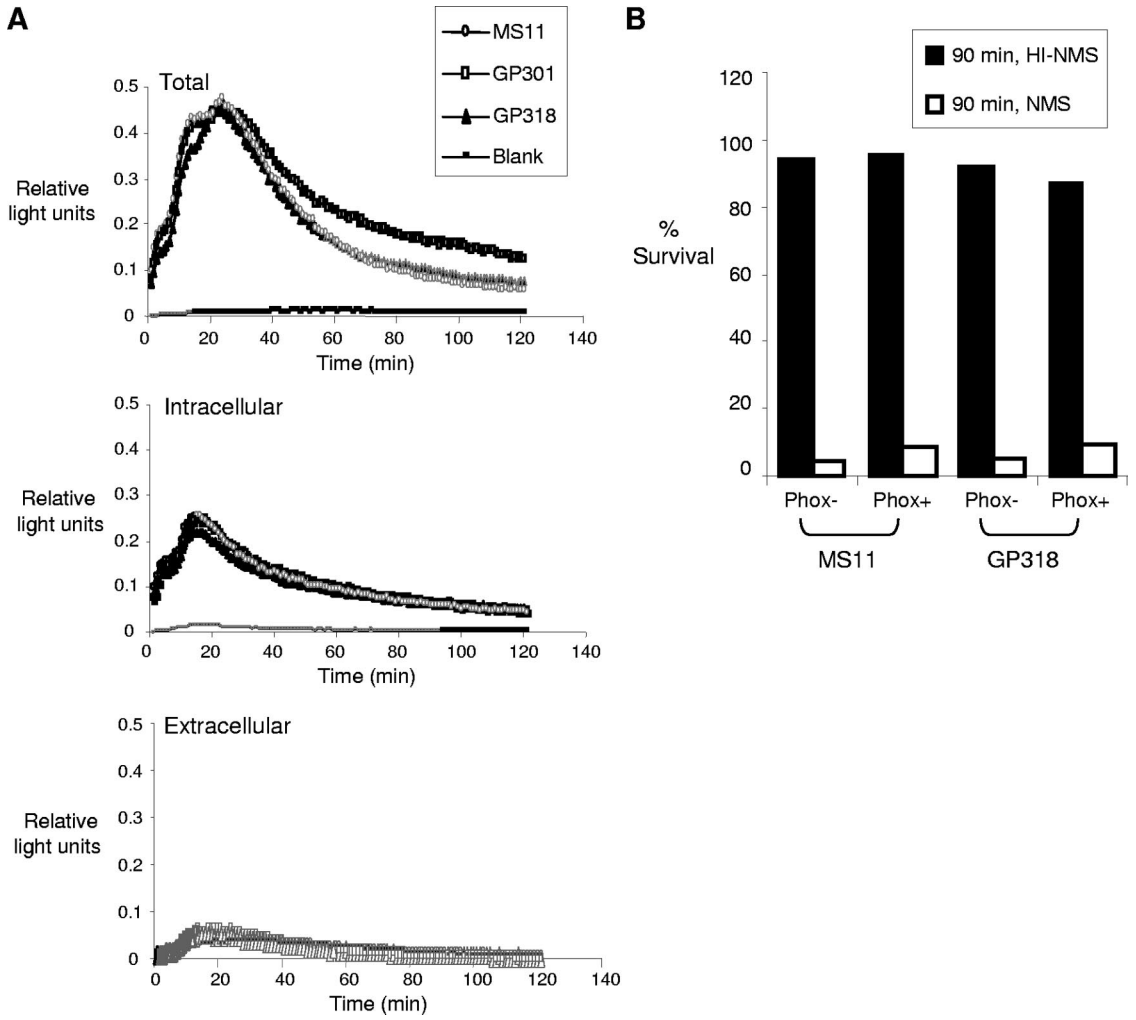

FIG. 6. Interactions of wild-type and mutant gonococci with murine PMNs. (A) CL response of PMNs from BALB/c mice after incubation with wild-type MS11, *kat* mutant GP301, and the *mntC kat ccp* mutant GP318. The total (top panel), intracellular (middle panel), and extracellular (bottom panel) CL responses upon exposure to bacteria (with no added phorbol myristate acetate) were measured as described previously (4, 58). (B) Opsonophagocytic killing of wild-type or GP318 (*mntC kat ccp*) mutant gonococci by PMNs from C57BL/6J (Phox-sufficient) and B6.129S6-Cybb^{tm1Din}/J (Phox-deficient) mice. Results are expressed as percent survival. The experiment was performed twice and the results were similar. HI-NMS, heat-inactivated normal mouse serum; NMS, normal mouse serum.

an *mntC* single mutant. Neither the *mntC* nor *mntC kat ccp* mutant showed increased susceptibility killing by PMNs from normal mice, and the *mntC kat ccp* mutant was not less susceptible to PMNs from mice that produce a defective NADPH oxidase. We further ruled out the possibility that inactivation of *mntC* increases susceptibility to phagocyte-derived ROS during genital tract infection by performing infection studies in Phox-deficient mice.

The basis for the attenuated phenotype of the MS11 *mntC* mutant in vivo is not known. Unfortunately we have been unable to introduce a wild-type copy of the *mntC* gene into the mutants GP304 and GP318, and therefore we have not definitively confirmed that the disadvantage shown in vivo is due to the absence of MntC. An impaired capacity to transport manganese and zinc (7, 28) through the MntABC system may impose an in vivo growth defect to the *mntC* mutant, since such metals may be needed as cofactors for enzymes that are important for gonococcal survival in vivo. We did not observe a difference in growth between wild-type FA1090 and the mutants that lack *mntC* when cultured in GCB or on GC agar. This result is in contrast to the report by Tseng et al. (52) in which an *mntC* mutant had a reduced growth rate when cultured on medium that had a fivefold-lower concentration of manganese than GC agar. It is unlikely that supplemented GC agar or broth provides the same balance of minerals found in the genital tract, and therefore we cannot rule out that reduced growth of the *mntC* mutant in vivo is responsible for the observed attenuated phenotype.

Interestingly, Mn^{2+} uptake mutants of *Streptococcus pneumoniae* (28a) were attenuated in murine respiratory and/or systemic infection models, the basis of which is not yet known. Epithelial cells can also produce ROS (39), and a gonococcal *mntC* mutant showed decreased intracellular survival in cervical epithelial cells (28).Other sources of ROS that might challenge gonococci that lack MntC include reactions with metal ions, including iron within heme-containing proteins released

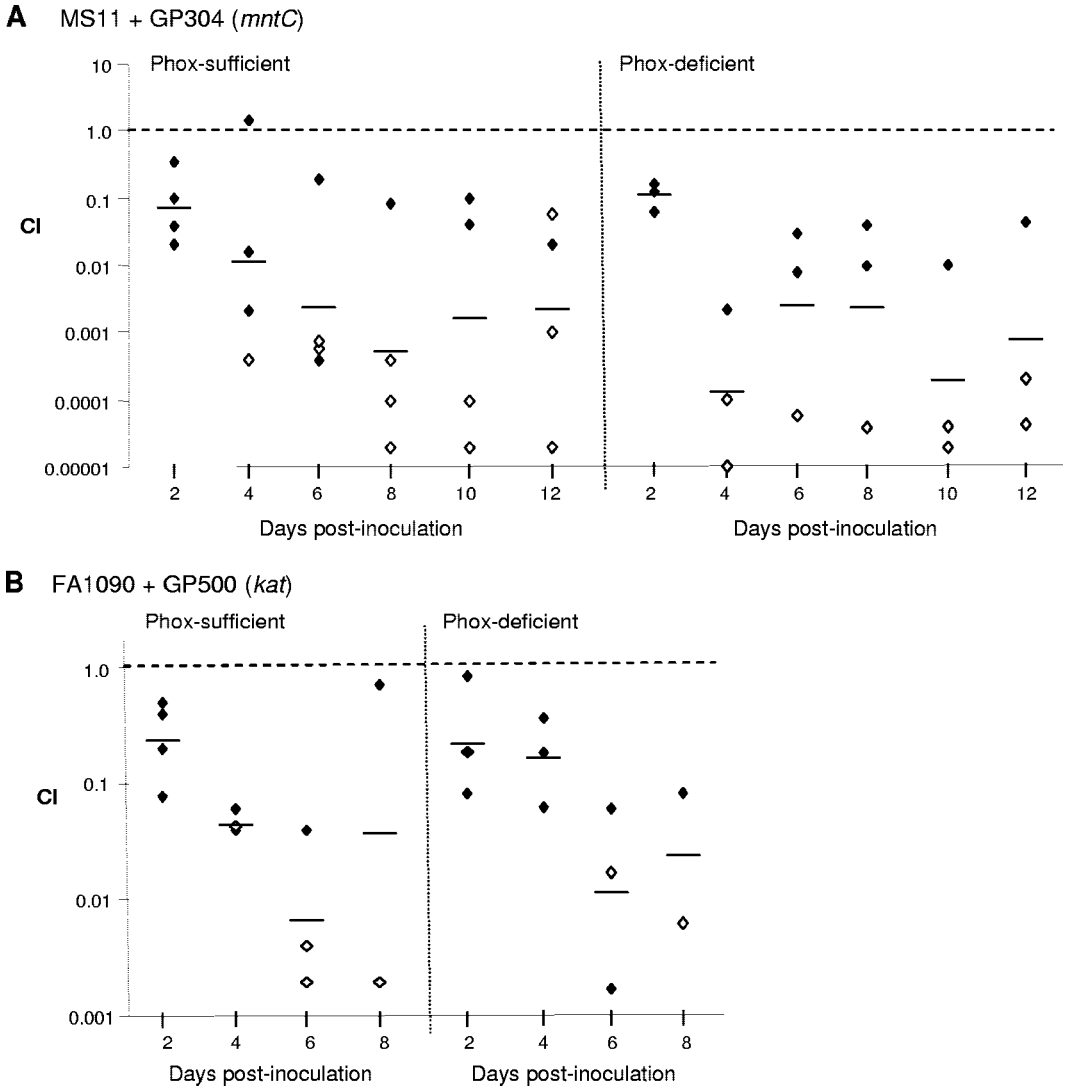

FIG. 7. The FA1090 *kat* mutant and MS11 *mntC* mutants are attenuated in Phox-deficient and wild-type C57BL/6J mice. FA1090 *kat* mutant GP500 and the MS11 *mntC* mutant GP504 were tested in B6.129S6-Cybb^{tm1Din}/J (Phox-deficient) and C57BL/6J (Phox-sufficient) mice by competitive infection with the respective parent strains. Experiments were performed as for Fig. 3 and 4. Results shown are the CI for individual mice at each time point. Horizontal bars represent the geometric means. A CI of <1.0 indicates a decrease in the ratio of mutant to wild-type gonococci compared to that of the inoculum and thus decreased fitness. Open symbols represent mice from which no mutants were recovered; the limit of detection (1 CFU per 100 μl of swab suspension) was used as the number of mutant CFU recovered in these cases.

from cells (10), and H_2O_2-producing commensal flora (14). We believe the latter explanation is unlikely since we rarely isolate H_2O_2-positive commensal vaginal bacteria from mice and because we have shown H_2O_2-producing human lactobacilli do not challenge wild-type gonococci or mutants that lack catalase or catalase and Ccp in the mouse model (32). Finally, recent evidence suggests the gonococcal MntABC transporter plays a role in biofilm formation (28), and therefore, inactivation of *mntC* may affect colonization of *N. gonorrhoeae* in the mouse model. The mean CI in competitive infection experiments with wild-type and *mntC* mutant bacteria was not as dramatic as that obtained in experiments with wild-type and *kat ccp mntC* mutant gonococci, and thus it is possible that the *kat ccp mntC* mutant may have a greater in vivo survival disadvantage than that exhibited by the *mntC* single mutant. Competitive infections between the *mntC kat ccp* triple mutant and the *mntC* single mutant would allow investigation of this hypothesis. We did not pursue this line of investigation, based on our in vitro and in vivo evidence that none of these genes contributes to evasion of PMN killing.

An unexpected but interesting finding was the strain-specific attenuation associated with the *kat* gene. A catalase-deficient mutant of FA1090, but not MS11, resulted in an in vivo growth or survival disadvantage that was unrelated to increased susceptibility to the phagocytic respiratory burst. We previously reported that the FA1090 *kat* mutant was more susceptible to PMN killing than wild-type FA1090 (46). We have since been unable to reproduce these data, which we believe reflects the high degree of variability in the PMN killing assay. We also found no correlation between PMN influx, colonization load,

and clearance of the *kat* mutant bacteria in the previous study, a result which is consistent with PMN killing not challenging catalase-deficient gonococci in vivo. As with the MS11 *mntC* mutant, the reason for the attenuation of the FA1090 *kat* mutant is not known, but it could be due to increased susceptibility to ROS produced by sources other than phagocytes.

The demonstration that loss of catalase is attenuating in one strain but not another is intriguing. MS11 is a serum intermediate strain that was originally isolated from the endocervix of an uncomplicated infection (51), and FA1090 is a serum-resistant strain isolated from a case of disseminated gonococcal infection (9). Others have reported that strain FA1090 is more sensitive to H_2O_2 than strains F62 and 28BI but that the three different strains have similar levels of catalase activity (2). We found that exposure of MS11 and FA1090 bacteria to H_2O_2 resulted in similar zones of inhibition (data not shown) and that the level of catalase activity in the two strains was the same (Table 3). We have detected other strain differences in the mouse model. For example, mutation of the lactate permease gene (*lctP*) conferred a growth or survival disadvantage in the murine genital tract to strain F62 (15), but not strain MS11 (H. Wu and A. E. Jerse, unpublished data). These observations underscore the importance of interpreting results in the context of the strain being studied and are evidence that evolutionary differences that affect the impact of specific adaptation genes on bacterial growth or survival in vivo have occurred.

Finally, we have presented evidence here that MsrA/B, which acts to repair oxidative damage rather than directly neutralize oxidative factors, enhances gonococcal survival late during infection of BALB/c mice. This attenuation was not observed in C57BL/6 mice. We consider these results intriguing based on our recent discovery that *N. gonorrhoeae* induces a vaginal PMN influx and proinflammatory cytokines and chemokines in BALB/c but not C57BL/6 mice (M. Packiam, R. R. Ingalls, and A. E. Jerse, Abstr. 16th Int. Pathog. *Neisseriaceae* Conf., Rotterdam, Netherlands, abstr. P097, 2008). There may therefore be a link between the *msrA* phenotype and the inflammatory response. Studies with PMNs from normal and Phox-deficient mice did not support the hypothesis that phagocyte-derived ROS challenge the *msrA* mutant in vivo; however, this protein may have other protective roles. *Mycobacterium tuberculosis* MsrA conferred increased resistance to nitrosative stress to *msrA* mutant *E. coli* (49), and *Mycobacterium smegmatis* MsrA was implicated in increased survival of *M. smegmatis* within macrophages by a mechanism that does not involve direct protection from H_2O_2 or reactive nitrogen intermediates (12). Our recent demonstration that macrophages are recruited to genital tract tissue in mice infected with *N. gonorrhoeae* at later time points than those at which we detect PMNs (48) is potentially consistent with the delayed attenuation of the gonococcal *msrA* mutant. Alternatively, a deficiency in MsrA/B may have pleiotropic effects by causing the accumulation of many different functionally impaired proteins, including adhesins (57).

In summary, several reports have shown that gonococci are killed by PMNs, primarily by oxygen-independent mechanisms (5, 37, 42). It is still not known, however, how gonococci evade the phagocytic respiratory burst. Our study was not exhaustive, as other factors are reported to protect the gonococcus from ROS in vitro, including thiol-disulfide oxidoreductase (Sco) (41), azurin (59), bacterioferritin (8), and newly described peroxidase-induced genes of unknown function (50). It is conceivable that the layers of functional redundancy in this well-adapted pathogen may be too thick to strip away by genetic mutation. Alternatively, the gonococcus may utilize a novel mechanism of evading phagocytic ROS. Finally, our findings suggest there are interesting roles for the MntABC transporter and the MsrA/B protein in vivo that as of yet are undefined.

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