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# RecA and RadA Proteins of *Brucella abortus* Do Not Perform Overlapping Protective DNA Repair Functions following Oxidative Burst

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**Very little is known about the role of DNA repair networks in *Brucella abortus* and its role in pathogenesis. We investigated the roles of RecA protein, DNA repair, and SOS regulation in *B. abortus*. While *recA* mutants in most bacterial species are hypersensitive to UV damage, surprisingly a *B. abortus recA* null mutant conferred only modest sensitivity. We considered the presence of a second RecA protein to account for this modest UV sensitivity. Analyses of the *Brucella* spp. genomes and our molecular studies documented the presence of only one *recA* gene, suggesting a RecA-independent repair process. Searches of the available *Brucella* genomes revealed some homology between RecA and RadA, a protein implicated in *E. coli* DNA repair. We considered the possibility that *B. abortus* RadA might be compensating for the loss of RecA by promoting similar repair activities. We present functional analyses that demonstrated that *B. abortus* RadA complements a *radA* defect in *E. coli* but could not act in place of the *B. abortus* RecA. We show that RecA but not RadA was required for survival in macrophages. We also discovered that *recA* was expressed at high constitutive levels, due to constitutive LexA cleavage by RecA, with little induction following DNA damage. Higher basal levels of RecA and its SOS-regulated gene products might protect against DNA damage experienced following the oxidative burst within macrophages.**

*Brucella*, a gram-negative bacterium, is the causative agent of brucellosis or Malta fever, a worldwide zoonosis affecting humans as well as a broad host range of mammals from domesticated animals to marine mammals with differences in host specificity and different human pathologies (10). *Brucella* spp. form a monospecific genus that belongs to the α-2 group of proteobacteria (58). They are phylogenetically closely related to soil bacteria, pathogens, and photosynthetic bacteria, including *Agrobacterium*, *Sinorhizobium*, *Rickettsia*, and *Rhodobacter* (37). *Brucella* species are facultative intracellular pathogens that invade both professional phagocytes, like macrophages and neutrophils, and nonprofessional phagocytes. Residence in this intracellular niche requires mechanisms for coping with a very hostile environment. The antimicrobial defense mechanisms of phagocytes are considerable and varied, including exposure to degradative enzymes, nutrient deprivation, exposure to reactive oxygen intermediates (ROI), and exposure to reactive nitrogen intermediates (RNI) (3, 25, 29). Both ROI and RNI are toxic and unstable compounds that react with and damage many cellular components, including proteins, membranes, and nucleic acids (3, 20, 54).

Bacteria may respond with multiple and redundant repair pathways to cope with DNA damage, including the SOS system, which is one of the most important (17, 20, 34, 60). A major protein of this system is RecA, a highly conserved multifunctional enzyme with pleiotropic effects on numerous cellular processes, including the SOS response, recombinational repair, cell cycle regulation, SOS mutagenesis, and replication restart (17, 20, 27, 61). The regulatory mechanisms of the SOS system have been extensively studied and are now well understood in some enterics, especially in *Escherichia coli*. In most of the bacteria studied, the SOS response is controlled by two major proteins: RecA, a positive regulator, and LexA, a negative regulator. Dimers of the LexA repressor bind to target operator sequences, a consensus motif that is referred to as the SOS box, and binding by LexA represses transcription of these SOS genes (20, 33, 60, 61). To date, about 30 SOS genes in *E. coli* have been identified, including the *recA* and *lexA* genes (11, 19). Upon DNA damage, regions of single-stranded DNA, thought to be the "signal" for SOS induction, are generated due to stalled replication forks (20, 31, 50). RecA then polymerizes on the single-stranded DNA regions and attains an activated conformation (RecA*) (20, 50). When the LexA repressor binds to the RecA* nucleoprotein filament, the repressor undergoes a conformational shift that promotes its autoproteolysis into nonfunctional

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TABLE 1. Bacterial strain list

| Strain | Relevant genotype or description | Resistance marker(s) | Source or reference |
|---|---|---|---|
| *B. abortus* | | | |
| 2308 | Wild-type, virulent, smooth lipopolysaccharide | | R. M. Roop |
| MEK5 | *uvrA*::*cat* | Cam$^r$ | R. M. Roop |
| MEK12 | *recA*::*cat* | Cam$^r$ | R. M. Roop |
| CMR1 | 2308/pDECR038 | Gen$^r$ | This study |
| CMR3 | MEK12/pDECR038 | Gen$^r$ | This study |
| CMR4 | *radA*::*kan* | Kan$^r$ | This study |
| CMR8 | CMR4/pDECR050 | Amp$^r$ | This study |
| CMR10 | MEK12/pDECR034 | Amp$^r$ | This study |
| CMR11 | MEK12/pDECR050 | Amp$^r$ | This study |
| CMR16 | *recA*::*cat radA*::*kan* | Cam$^r$, Kan$^r$ | This study |
| CMR17 | *recA*::*cat radA*::*kan*/pDECR034 | Amp$^r$ | This study |
| CMR18 | *recA*::*cat radA*::*kan*/pDECR050 | Amp$^r$ | This study |
| *E. coli* | | | |
| SR2643[a] | Thr$^+$ | | 53 |
| SR2708[a] | *radA*::*kan* | Kan$^r$ | 53 |
| DE3992[a] | SR2708/pDECR046 | Kan$^r$, Amp$^r$ | This study |
| DE3993[a] | SR2708/pserB59-1 | Kan$^r$, Amp$^r$ | This study |
| DE3357[b] | *mal*$^+$ | | This study |
| DE1663[b] | Δ(*srlR-recA*)*306*::Tn*10* | Cam$^r$, Tet$^r$ | 23 |
| DE3534[b] | DE1663/p*BarecA* | Amp$^r$ | This study |

[a] These strains also have the established AB1157 genotype: *argE3 hisG4 leuB6* Δ(*gpt-proA*)*62 thr-1 thi-1 ara-14 galK2 lacY1 mtl-1 xyl-5 tsx-33 rfbD1 mgl-51 rpsL31 supE44 rac* F$^-$ λ$^-$.

[b] These strains also have the genotype *sulA211 ilv*(*Ts*) *thi-1 rpsL31 argE3 supE44 thr-1 hisG4 leuB6 galK2 malB*::Tn*9* Δ(*lac-gpt*)*U169* F$^-$ (λ *c*I Ind-1 *recAo*/*p*::*lacZYA*).

fragments (35). Following LexA cleavage, the pool of functional LexA repressor decreases and expression of the SOS genes is increased. Induction of the *E. coli* SOS genes ranges from 5- to 10-fold for genes such as *lexA*, *umuDC*, *uvrAB*, or *ruvAB*, 20- to 50-fold for *recA*, or >100-fold for the *sulA* gene (11, 17, 20, 61). Derepression of the SOS regulon results in increased DNA repair, pause of cell division, and error-prone DNA synthesis (17, 34, 60, 61). As DNA damage is repaired, the activating signal drops, the amount of RecA* declines, levels of intact LexA accumulate, and finally, repression of the SOS genes is reestablished (20, 34).

The *Brucella abortus recA* gene was originally cloned and sequenced by Tatum et al. (56), and the deduced protein sequence had 86% identity with the related bacterium *Sinorhizobium meliloti* RecA and about 70% identity with that of *E. coli* and other *Enterobacteriaceae* (15, 56). They constructed a *B. abortus recA* mutant that was sensitive to the chemical mutagen methyl methane sulfonate (MMS), indicating that RecA functions have an important role in allowing this organism to survive exposures to DNA damage (56). However, colonization studies with BALB/c mice showed that even though the numbers of bacteria per spleen were approximately 100-fold lower than in the wild type, the *recA* mutant persisted as long as the wild type. Thus, Tatum et al. (56) concluded that although inactivation of RecA may attenuate *B. abortus*, RecA is not crucial for persistence in mice.

The precise role(s) of RecA in *Brucella* virulence and protection against the damage from the oxidative burst within macrophages remains unclear. Buchmeier et al. demonstrated that *Salmonella* RecA was essential for full virulence in vivo and was required to survive the oxidative burst (7). In this report, we investigated whether the RecA protein of *B. abortus*, which lives in a similar niche as *Salmonella*, had similar requirements. First, we were surprised to discover that *recA* mutants were not hypersensitive to UV damage, and we investigated whether a related DNA repair protein, RadA, might compensate for the loss of RecA in *B. abortus*. Next, we discovered that *B. abortus* RecA protein was constitutively activated (RecA*) for LexA cleavage without the normal requirements for DNA damage. We speculate that high constitutive SOS expression might confer a protective role against the damage experienced within macrophages. These observations suggest that although many aspects of the *Brucella* DNA repair systems are similar to those of other model bacterial systems, key components of *B. abortus* repair networks have diverged.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and culture conditions.** Bacterial strains, phages, and plasmids used in this study are listed in Tables 1 and 2 with their relevant features. Bacterial strains were constructed by using standard methods of plasmid transformation (36, 48). All *Escherichia coli* K-12 strains were maintained and grown in Luria-Bertani (LB) media (36). The appropriate antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 80 μg/ml; chloramphenicol, 20 μg/ml; gentamicin, 10 μg/ml; tetracycline, 25 μg/ml. All *Brucella* strains were grown at 37°C under 5% $CO_2$ either in brucella broth (Difco), on Schaedler agar (Difco) with 5% horse serum, or on Trypticase soy agar (TSA; Difco Industries) supplemented or not with 5% defibrinated bovine blood. Ampicillin, kanamycin, gentamicin, and chloramphenicol were added to these culture media at final concentrations of 50 μg/ml, 45 μg/ml, 10 μg/ml, and 5 μg/ml, respectively. All work with live *B. abortus* was performed at biosafety level 3 as per CDC and USDA select agent regulations.

**β-Galactosidase measurement assays.** In *E. coli*, the expression of the chromosomal *E. coli recAO*/*P-lacZ* reporter was performed essentially as described by Hintz et al. (23), except that β-galactosidase activity was measured following 2 h of mitomycin *C* (MC) exposure. In *B. abortus*, expression of β-galactosidase activity from a plasmid-borne *Brucella recAO*/*P-lacZ* translational fusion (pDECR38) was essentially as described above and by Miller (36). Here *B. abortus* cultures of about $1.5 \times 10^8$ bacteria/ml were split, and then half were exposed to 0.5 μg/ml MC for 2 h and half were not exposed.

**Strain construction and recombinant DNA techniques.** For construction of *B. abortus* strain MEK12, a 373-bp SalI-EcoRV internal fragment was removed

TABLE 2. Plasmid list

| Plasmid | Relevant genotype or description | Resistance marker(s) | Source or reference |
|---|---|---|---|
| Cloning vectors | | | |
| pBBR1MCS-4 | Broad-host-range plasmid | $Amp^r$ | 30 |
| pBBR1MCS-5 | Broad-host-range plasmid | $Gen^r$ | 30 |
| pCR2.1 | | $Kan^r$, $Amp^r$ | Invitrogen |
| pUC4K | ColE1 ori | $Kan^r$, $Amp^r$ | 59 |
| pUC19 | ColE1 ori | $Amp^r$ | 59 |
| Recombinant plasmids | | | |
| pLKC481 | pNM480::Tn*5* | $Kan^r$, $Amp^r$ | 57 |
| pserB59-1 | *smp*$^+$ *serB*$^+$ *sms*$^+$ | $Amp^r$ | 40 |
| p*BarecA* | pUC12 ClaI::*BarecA* | $Amp^r$ | 56 |
| pMEK10 | pUC12 ClaI::*BarecA*Δ(SalI-EcoRV)::*cat* | $Amp^r$, $Cam^r$ | This study[a] |
| pDECR018 | pDECR017 HindIII::*BarecAO*/*P* | $Kan^r$ | This study[a] |
| pDECR034 | pBBR1MCS-4 Δ(BamHI-PstI)::*BarecA* | $Amp^r$ | This study[a] |
| pDECR038 | pBBR1MCS-5 PstI::Φ(*BarecAO*/*P-lacZ*) ΔNcoI ΔBstBI | $Gen^r$ | This study[a] |
| pDECR045 | pCR2.1::*BaradA* | $Amp^r$, $Kan^r$ | This study[a] |
| pDECR046 | pUC19 Δ(BamHI-XbaI)::*BaradA* | $Amp^r$ | This study[a] |
| pDECR048 | pUC19 Δ(BamHI-XbaI)::*BaradA*Δ(EcoRV-NaeI) | $Amp^r$ | This study[a] |
| pDECR049 | pUC19 Δ(BamHI-XbaI)::*BaradA*Δ(EcoRV-NaeI) NcoI::*kan* | $Amp^r$, $Kan^r$ | This study[a] |
| pDECR050 | pBBR1MCS-4 Δ(BamHI-XbaI)::*BaradA* | $Amp^r$ | This study[a] |

[a] Descriptions for plasmid constructions are given in Materials and Methods.

from p*BarecA* (56) and replaced by a SalI-SmaI *cat* gene to generate pMEK10. Plasmid pMEK10 was then introduced into *B. abortus* 2308 by electroporation to disrupt the *recA* gene, and a chloramphenicol-resistant recombinant was selected and then screened for ampicillin-sensitive isolates (products of double recombination events). For construction of a translational fusion of the *B. abortus recA* promoter to the *lacZ* reporter gene, plasmid pDECR038 was generated as follows: a 4.0-kb HindIII fragment from p*BarecA* (56) containing the *recAO*/*P* was cloned upstream of the promoterless *lacZ* gene in an $Amp^s$ derivative of pLKC481 containing a *lacZY*-$Kan^r$ cassette (57). The 9.4-kb PstI fragment carrying the *B. abortus recAO*/*P*::*lacZY* translational fusion was cloned into the broad-host-range vector pBBR1MCS-5 (30). To delete the *lacO*/*P* in the vector so that the *recA-lacZ* gene fusion is transcribed only from the *B. abortus recA* promoter, an NcoI 2.7-kb fragment was deleted. Finally, an additional 2.4-kb BstBI fragment was deleted to remove a kanamycin resistance gene, yielding the plasmid construct pDECR038. This plasmid was introduced into *B. abortus* 2308 and MEK12 by electroporation. For construction of *B. abortus* strain CMR4, the *radA* gene was amplified from genomic DNA of *B. abortus* 2308 using primers 5′-GGCCATGGTTCTCGTCATCGACTATGTGC-3′ and 5′-GCGAACCATC GCCAGTACGGC-3′ corresponding to genome sequences located 500 bp upstream and 200 bp downstream of the *radA* gene, respectively. The temperature cycle regimens were 1 cycle of denaturation at 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min and a final elongation step at 72°C for 7 min. The amplified 2,101-bp fragment was then cloned into the pCR2.1 vector (Invitrogen). The structure of the cloned *radA* gene was confirmed by restriction analysis and by nucleotide sequencing of the gene. The *radA* gene was excised with BamHI and XbaI restriction enzymes and cloned into pUC19, resulting in plasmid pDECR046. To delete a portion of the *radA* gene, an internal NaeI-EcoRV 644-bp fragment was first removed, resulting in pDECR048. Next, NcoI linkers were added to the kanamycin resistance gene, from pUC4K (59), and inserted in the NcoI restriction site, located 41 bp downstream from the above NaeI-EcoRV deletion, yielding pDECR049. The gene replacement construct was introduced into *B. abortus* 2308 by electroporation, selection for a kanamycin-resistant recombinant was made, and then screening was done for ampicillin-sensitive isolates; one confirmed construct was designated CMR4. Plasmid pMEK10 was then introduced into CMR4 by electroporation to disrupt the *recA* gene and a chloramphenicol-resistant, ampicillin-sensitive recombinant was identified and designated CMR16. Gene replacement in *B. abortus* was confirmed both by PCR amplification and by Southern blotting for all mutants (data not shown). For construction of plasmids pDECR034 and pDECR050, a 5.3-kb BamHI-PstI fragment from p*BarecA* containing the *B. abortus recA* gene and the BamHI-XbaI 2.1-kb fragment containing the *B. abortus radA* gene were cloned into the vector pBBR1MCS-4.

**Bacterial sensitivity to chemical mutagens by Kirby-Bauer disk diffusion assays (32).** *Brucella* cells were obtained either following overnight incubation in brucella broth or following 72 h of incubation on plates at 37°C with 5% $CO_2$ where the cells were scraped and resuspended in 1 ml of phosphate-buffered saline (PBS). The cultures were adjusted to an optical density at 600 nm of 0.2, and 100 μl of the bacterial suspensions was spread onto plates. An aliquot of 10 μl of 10% MMS or 15 μl of 3% $H_2O_2$ was loaded onto a sterile disk (S&S filter paper, 1/4 in.; ISC Bioexpress). The zones of inhibition on either Shaedler agar plates (for MMS) or TSA plates (for $H_2O_2$) were measured after 72 h of incubation at 37°C with 5% $CO_2$.

**UV sensitivity assay.** UV survival estimates were based on colony counts and were conducted essentially as described by Ennis et al. (17). *Brucella* cells were grown to mid-log phase and diluted 10-fold in 10 mM $MgSO_4$ to avoid shielding and then irradiated at the desired UV doses. Irradiated cells were serially diluted and plated on Shaedler agar plates under subdued lighting to avoid photoreactivation. Colony counts were performed after 72 h of incubation at 37°C with 5% $CO_2$. The surviving fraction (S/So) is expressed as the quotient of the viable count at a specific UV dose (S) (expressed in J/m$^2$) divided by the titer of bacteria without UV exposure (So).

**Isolation and infection of peritoneal macrophages.** Macrophages were harvested from the peritoneal cavities of 8-week-old female BALB/c mice pretreated by an injection of 1 ml of 5% Proteose peptone 1 week prior to the macrophage harvest. The macrophages were washed with RPMI plus 5% fetal calf serum (FCS) supplemented with 5 units of heparin per ml. Pooled macrophages were cultivated in 96-well plates at a concentration of $7.5 \times 10^4$ per well in 200 μl of RPMI plus 5% FCS at 37°C with 5% $CO_2$. Macrophages were exposed overnight to RPMI plus 5% FCS supplemented or not with either 5 U of recombinant gamma interferon (IFN-γ) (Roche, Indianapolis, IN). Macrophages were washed three times with 100 μl PBS plus 0.5% FCS. *Brucella* cells were opsonized for 30 min with a subagglutinating dilution (1:500) of hyperimmune BALB/c mouse serum in RPMI plus 5% FCS. Opsonized cells were added to the macrophages at a multiplicity of infection of 100 and were allowed to be phagocytized for 2 h at 37°C. Then the culture medium was replaced by RPMI plus 5% FCS supplemented with 50 μg/ml gentamicin for 1 h to kill the extracellular bacteria. The macrophages were then washed three times with 100 μl of warm PBS plus 0.5% FCS and lysed with 0.1% deoxycholate. The intracellular bacterial cells were serially diluted in PBS and plated on *Brucella* agar with the appropriate antibiotic. This time point was designated "time zero." For the remaining wells, the medium was replaced by RPMI plus 5% FCS supplemented with 20 μg/ml gentamicin and incubated overnight at 37°C with 5% $CO_2$. Washes, lysis, and bacterial plating were repeated at the 48-h time point. The percent survival was expressed as the quotient of the titer of intracellular bacteria at a specific time divided by the titer of intracellular bacteria present in macrophages at "time zero" multiplied by 100.

**Statistical analysis.** Statistical analyses were performed using the Student *t* test.

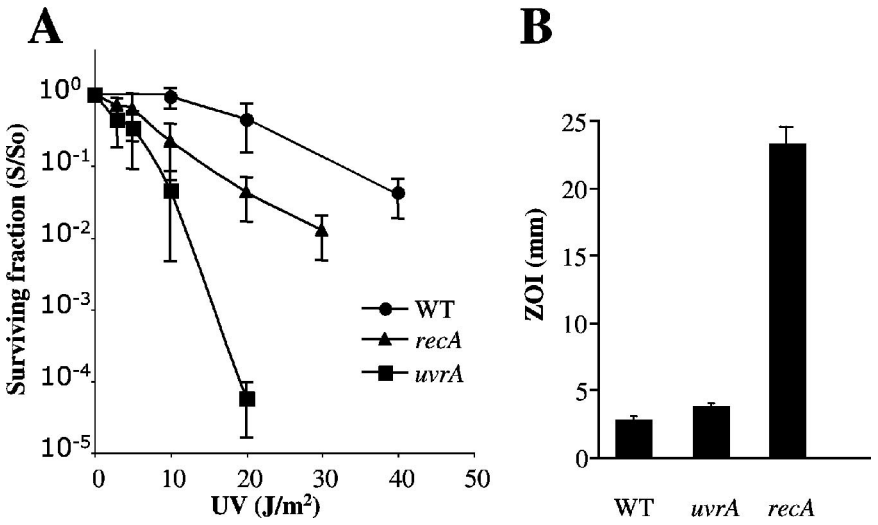


FIG. 1. The *B. abortus recA* mutant is not hypersensitive to UV damage. (A) Survival plots of *B. abortus* 2308 (wild type [WT]), MEK12 (*recA*::*cat*), and MEK5 (*uvrA*::*cat*) are represented. (B) Suspensions of the same strains were spread onto plates, and then sterile disks were placed in the centers of the plates. MMS was loaded onto disks, and zones of inhibitions (ZOI) were measured after incubation at 37°C with 5% $CO_2$ for 72 h. Each value is the mean ± standard deviation of results from at least three independent experiments.

## RESULTS

***B. abortus recA* mutant exhibits a modest sensitivity to UV irradiation.** The responses of *B. abortus recA* and *uvrA* repair mutants to UV damage were compared to that of the parental wild-type strain. As expected, both of these mutants exhibited greater sensitivity than the wild-type strain (Fig. 1A). However, it was unexpected that the *recA* mutant conferred only a modest sensitivity to UV, substantially less sensitive than the *uvrA* mutant. Inactivation of *recA* in nearly all bacterial species examined produces mutants that are hypersensitive to DNA damage because RecA plays central roles in a number of important processes that respond to DNA damage, including regulation of SOS repair genes, resumption of replication, and homologous recombination (17, 31, 46, 61). Mutant strains carrying inactivated *uvrA* genes are typically less sensitive than *recA* mutants because there is only the loss of the nucleotide excision repair system, just one subset of the larger repair networks (17, 20, 22, 46). The *B. abortus recA* disruption mutation was designed to be a null allele; this was achieved by first deleting a central 373-bp interval within the gene, known to code for components of RecA protein that are essential for activity (15, 27), and then replacing that interval with a chloramphenicol resistance cassette. The resulting *recA*::*cat* disruption mutant would be expected to produce only a nonfunctional N-terminal third of the RecA protein (27, 46). Indeed, functional studies of this *recA*::*cat* construct found it to be completely defective for recombination and LexA cleavage activities in *E. coli* (data not shown). The modest sensitivity of the *recA* mutant suggested that *B. abortus* RecA had a less prominent role in repair of UV damage than its homologues in other bacteria. However, the resistance to UV by the *recA* mutant strain did not extend to all other mutagens, as was described by Tatum et al. (56), *B. abortus recA* mutants were hypersensitive to the potent mutagen MMS, a DNA methylating agent (Fig. 1B). The *Brucella uvrA* mutant was resistant to MMS; this resistance was not unexpected, since in other bacteria, the AlkA protein of the adaptive response recognizes and removes the bases with MMS adducts, not the UvrABC excision repair complex (20). To account for the UV resistance of the *recA* mutant, we hypothesized that *B. abortus* might express multiple RecA proteins. Both *Myxococcus xanthus* and *Bacillus megaterium* were found to carry two *recA* genes (38, 42). The previous genetic analyses of *B. megaterium* had parallels to our current studies on *B. abortus*, since the *recA* mutant also exhibited moderate UV sensitivity compared to an isogenic *uvrA* mutant (16, 38).

**Elevated SOS expression due to constitutive activation of the *B. abortus* RecA protein.** To better characterize the *B. abortus* RecA protein and its roles in SOS regulation, we monitored the expression from a fusion of the *B. abortus recA* operator/promoter region with the *lacZ* gene in both the wild type and in the mutants. As shown in Fig. 2, the wild-type strain exhibited a high basal level of β-galactosidase expression followed by a small twofold increase following MC-inducing treatment. This induction was rapid and quickly reached a peak, since maximum activity was observed following 30 min of MC exposure and no increase occurred over 24 h (data not shown). High constitutive expression was also observed in the *uvrA* mutant (data not shown). Interestingly, the *recA* mutant exhibited very low levels of *recA-lacZ* expression with and without MC induction. The observations that high levels of *recA-lacZ* expression were seen with little induction in both wild-type and *uvrA* strains and that this expression is greatly reduced by inactivation of the *recA* gene indicated that the *Brucella* RecA protein was constitutively activated. These results also demonstrated that the SOS regulatory network of *Brucella* is similar to that of most other bacterial species and that RecA is the principal positive regulator of the SOS regulon. However, unlike most species examined, the RecA* activity for LexA cleavage occurs without the normal requirements for inducing treatments. To further investigate this spontaneous RecA* activity, the *B. abortus recA* gene was introduced

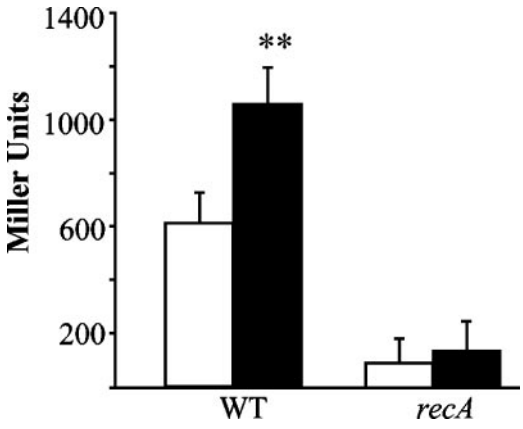


FIG. 2. Effect of DNA damage on *recA* expression in *B. abortus*. Expression of *recA* was measured by monitoring the activity of *recAO*/*P*::*lacZ* reporter on plasmid pDECR038 in *B. abortus* 2308 (wild type [WT]) and MEK12 (*recA*::*cat*) following 2 h of growth in *Brucella* broth with 0.5 μg/ml mitomycin C (black bars) or without damage (white bars). β-Galactosidase activities were measured from one representative experiment, and the error bars represent standard deviations. **, $P < 0.01$.

into an *E. coli* strain carrying an *E. coli recA-lacZ* fusion and a deletion that removes the resident *recA* gene (23). We observed that the *Brucella* RecA also conferred high constitutive expression of an *E. coli* SOS gene fusion, followed by a twofold induction (as opposed to a sixfold induction with *E. coli recA*) (Fig. 3). These data confirmed that the *B. abortus* RecA protein itself was constitutively activated and it is not being activated by some other component in the *B. abortus* cell.

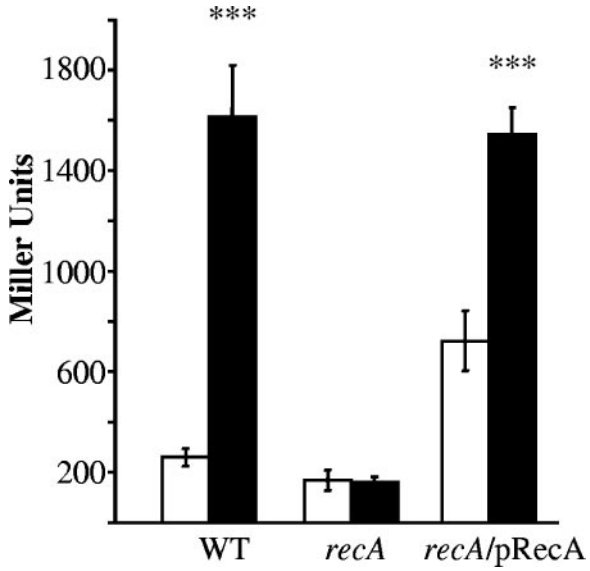


FIG. 3. Constitutive RecA activation of the *B. abortus* RecA protein in *E. coli*. Expression of an *E. coli recA*::*lacZ* reporter fusion in DE3357 (wild type [WT]), DE1663 (Δ*recA*), and DE3534 (Δ*recA*/p*BarecA*) following 2 h of growth in broth with 0.5 mg/ml mitomycin C (black bars) or without exposure (white bars). The β-galactosidase activity represents the mean value of results from at least four independent experiments, and the error bars represent standard deviations. ***, $P < 0.01$.

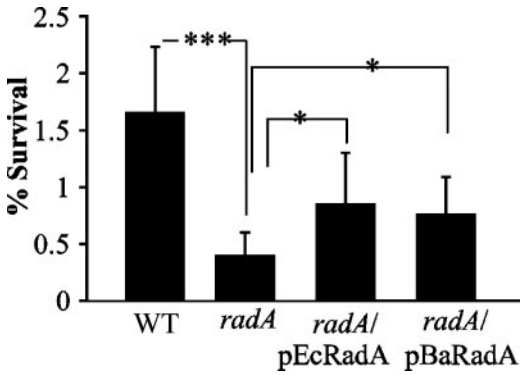


FIG. 4. Genetic complementation of an *E. coli radA* mutant by the *B. abortus radA* gene. Strains SR2643 (wild type [WT]), SR2708 (*radA*::*kan*), DE3993 (*radA*::*kan*/pEcRadA), and DE3992 (*radA*::*kan*/pBaRadA) were exposed for 20 min to a pulse of 5 μg/ml of MC, washed, and plated for viable counts. The graph shows the mean values of results from at least three independent experiments. *, $P < 0.05$; ***, $P < 0.01$.

**Attempts to identify additional *recA*-like genes in *B. abortus*.** As noted above, we hypothesized that some other RecA-like function is present in *Brucella* to compensate for the loss of RecA protein in the *recA* mutant. To identify this postulated repair function, we attempted to clone or detect other *recA*-like genes in *Brucella*. We employed several established strategies such as functional complementation, used previously to clone the *B. abortus recA* gene (56), Southern blotting, and PCR with degenerate primers, which have been used to clone *recA* genes from bacteria related to *Brucella* spp. such as *Rickettsia prowazekii* and *Rhodopseudomonas* (9, 13). However, all of our efforts failed to identify a "second" *recA* gene. During the course of this study, the genome sequences for *Brucella melitensis* 16M, *Brucella suis* 1330, and finally, *Brucella abortus* 2308 became available, all of which confirmed the presence of only one unique *recA* gene in each of these pathovars. To find a protein that might have functions similar to those of the RecA protein (and primary sequence), we then performed a BLASTP search using the *B. abortus* RecA amino acid sequence as a query, which directed our attention to RadA, the closest match in these genomes (*E* value of 0.012) (1). An alignment of *B. abortus* RadA and RecA proteins showed the greatest homology in the N-terminal and middle regions (data not shown), as was observed for both of these *E. coli* proteins by Neuwald et al. (40, 41). Since the *E. coli* RadA protein has been implicated in DNA repair (4, 41, 49) and the RadA protein was the closest match, we proceeded to investigate if this protein from *B. abortus* could functionally replace RecA.

***B. abortus radA* can complement an *E. coli radA* mutant.** We cloned the *radA* gene from *B. abortus* to test whether this RadA protein can complement an *E. coli radA* mutant. The *E. coli radA* mutant was fivefold more sensitive to this MC exposure, and this sensitivity was partially reversed with the introduction of a plasmid carrying the *B. abortus radA*, indicating some complementation in *E. coli* (Fig. 4). Interestingly, the plasmid carrying the *E. coli radA* gene also yielded a partial reversal of MC sensitivity. Although full resistance was not restored to this *radA* mutant by either of these *radA* genes, the observation that the *B. abortus radA* restored MC resistance to

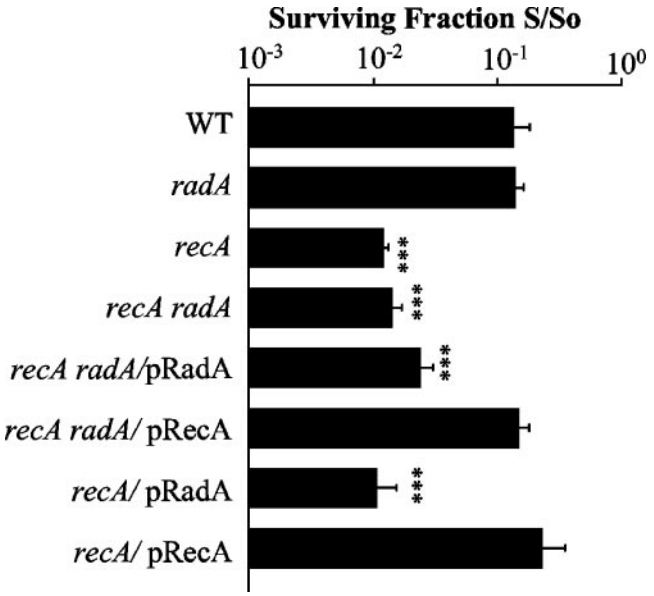


FIG. 5. *B. abortus recA* and *radA* genes do not perform redundant UV repair activities. *B. abortus* 2308 (wild type [WT]), CMR4 (*radA::kan*), MEK12 (*recA::cat*), CMR16 (*recA::cat radA::kan*), CMR16 mutant complemented by a plasmid-borne cloned copy of *radA* (CMR18) or *recA* (CMR17), and the *recA*::*cat* mutant complemented by a plasmid-borne cloned copy of *radA* (CMR11) or *recA* (CMR10) were exposed to 20 J/m$^2$ of UV irradiation. Colony plate counts were performed after incubation at 37°C with 5% $CO_2$ for 72 h. The graphs show the mean values of results from at least three independent experiments. ***, $P < 0.001$.

the same level as the *E. coli* gene suggested that the *B. abortus radA* clone was functional and that RadA could be involved in repairing MC-induced damage.

**RadA does not compensate for the inactivation of RecA.** We have conducted a number of experiments to test the hypothesis that the RadA protein might exhibit similar functions of RecA in *B. abortus* and thus account for the modest sensitivity to UV irradiation of the *recA* mutant. One approach was to compare the sensitivities of *recA* and *radA* single mutants of *B. abortus* to that of a strain in which both *recA* and *radA* genes were inactivated. We reasoned that if RecA and RadA proteins had redundant activities, the *recA radA* double mutant of *B. abortus* would be hypersensitive compared to either of the single mutants. We compared the responses of these single and double mutant strains following exposure to one modest UV dose (20 J/m$^2$). The *recA* mutant was about 10-fold more sensitive than the *radA* mutant or wild type with UV radiation (Fig. 5). The *recA radA* double mutant was as UV sensitive as the *recA* mutant, and introduction of a plasmid-encoded RecA in either the *recA* or double mutant was able to restore a resistant phenotype to UV exposure that approached the level of the parental wild type (Fig. 5). The observation that the *recA* mutant and the *recA radA* double mutant confer essentially the same sensitivity to DNA damage argues against the notion that RadA protein of *B. abortus* can function in place of RecA protein.

**Response of *B. abortus recA* mutant to $H_2O_2$.** We further evaluated the response of the repair mutants to $H_2O_2$ damage, an oxidative stressor that would be encountered within macrophages. The *recA* mutant displayed a slight sensitivity to $H_2O_2$ killing compared to both the repair mutants examined (*radA* and *uvrA*) and the wild-type parental strains (Fig. 6 and data not shown). Full resistance to $H_2O_2$ was restored upon reintroduction of the *recA* gene on a multicopy plasmid.

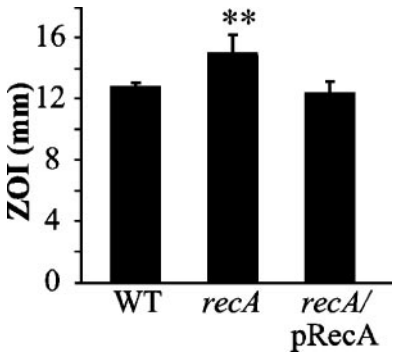


FIG. 6. Inactivation of the *recA* increases the sensitivity of *B. abortus* to hydrogen peroxide: $H_2O_2$-mediated killing of *B. abortus*. *B. abortus* 2308 (wild type [WT]), MEK12 (*recA*::*cat*), and CMR10 (*recA*::*cat*/pRecA) were spread on TSA plates, and 15 ml of 3% $H_2O_2$ was loaded onto a sterile disk and incubated at 37°C with 5% $CO_2$ for 72 h. The graph shows the mean values of the zones of inhibition (ZOI) of results from at least three independent experiments. **, $P < 0.01$.

**RecA protein is important for survival of *B. abortus* in macrophages.** We investigated the significance of DNA repair in *B. abortus* infection (56) and, more specifically, the implication of RecA in the repair of DNA damage within murine macrophages. As shown in Fig. 7, after 48 h of infection, intracellular survival of the *recA* mutant was significantly attenuated, about fourfold more sensitive than the wild type. With an activating pretreatment of macrophages by IFN-γ, the *recA* mutant became significantly more sensitive to the bactericidal activities of the macrophages. These data indicated that the *B. abortus* RecA plays a significant role in conferring resistance to the lethal products released during the oxidative burst of activated

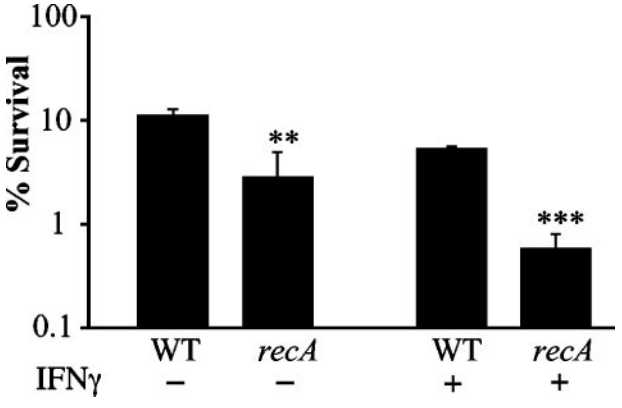


FIG. 7. Inactivation of the *recA* gene increases the sensitivity of *B. abortus* to macrophages. Viable counts of intracellular *B. abortus* strains 2308 (wild type [WT]) and MEK12 (*recA*::*cat*) following incubation within peritoneal murine macrophages are shown. Macrophages were preincubated in plain culture medium (−) or 5 U of IFN-γ (+). Three wells were evaluated at each time point for every strain tested. **, $P < 0.01$; ***, $P < 0.001$.

macrophages and were consistent with the observation that the *recA* mutant exhibited some hydrogen peroxide sensitivity in vitro (Fig. 6).

## DISCUSSION

Very little is known about the DNA repair networks of the intracellular pathogen *Brucella abortus* and the role(s) that repair plays in pathogenesis. Major goals of this project included better characterization of the DNA repair networks of *B. abortus*, SOS regulation of repair, and evaluation of the requirement(s) for repair in surviving within host macrophage cells. Our characterizations were initially guided by the assumption that the *B. abortus* DNA repair networks would likely share many features of the networks present in model systems like *E. coli*. Indeed, our characterizations revealed a number of similarities, but two major unforeseen differences were uncovered in the *B. abortus* repair systems. First, we observed nearly maximal expression of an SOS gene even without exogenous DNA damage. Second, a *recA* mutant of *B. abortus* was surprisingly resistant to UV irradiation compared to most other bacterial species lacking functional RecA protein.

We investigated the damage-inducible DNA repair systems or SOS repair of *B. abortus*. The expression levels from plasmid-encoded *recAO/P::lacZ* translation fusion strains were compared with or without DNA damage. High levels of *recA-lacZ* expression were measured in the unexposed wild-type *B. abortus* cells, and only a modest twofold induction was seen following exposure to MC. In contrast, *E. coli* RecA was induced about 10-fold or greater after exposure to MC or other mutagens (6, 28, 47). High basal *recA* expression was observed in the *uvrA* repair mutant (data not shown), but expression was reversed in the *B. abortus* mutant strain lacking RecA and no increase of *recA-lacZ* activity was seen in the same mutant with MC damage. Functional studies with *E. coli* SOS fusion strains also demonstrated that the *B. abortus* RecA was constitutively activated for cleavage of the LexA repressors from both *E. coli* and *B. abortus* (Fig. 2 and 3 and data not shown). These results indicated the following. First, as in most bacteria, the SOS system of *Brucella* is positively regulated by RecA. Second, the high basal *recA-lacZ* expression was the result of constitutive RecA activation (RecA*), leading to the cleavage of LexA without the normal requirements for an inducing treatment. Indeed, comparisons of the constitutive RecA* properties of the wild-type *B. abortus recA* in *E. coli* were similar to *E. coli* cells carrying mutant alleles such as *recA432* and *recA718* (17, 18, 20, 61; also data not shown).

High basal RecA levels in *B. abortus* with a small induction would be formally similar to other bacteria like *Mycobacterium tuberculosis* and *Deinococcus radiodurans* (5, 39, 44). Although the lower basal RecA levels are adequate for *E. coli* and other enteric bacteria to efficiently catalyze recombination and respond to DNA damage (20, 22), these bacteria with high basal RecA levels live in harsh environments. High basal RecA levels might reflect an adaptation to hostile environments compared to the SOS systems of the enterics that evolved in comparatively benign environments. The enteric pathogen *Salmonella enterica* serovar Typhimurium is like *Brucella* and survives within the hostile phagolysosomes of activated macrophages; however, *S. enterica* serovar Typhimurium also expresses low basal RecA levels that are comparable to those of *E. coli* (45). However, since *Brucella* is a much slower-growing organism than *Salmonella*, a greater accumulative mutagenic assault to the genome would be expected before each doubling. We suggest that high basal levels of RecA might allow slow-growing intracellular pathogens like *Brucella* and *Mycobacterium* to compensate for the extended exposure times between cell divisions. Mutational analyses of *D. radiodurans* DNA repair networks revealed that although LexA was cleaved by RecA, the *recA* gene was regulated by a LexA-independent mechanism (5, 39). A second positive regulator of DNA repair, IrrE, was uncovered that stimulates the transcription of the *recA* following DNA damage, and it is the action of this second regulator that contributes to high basal RecA levels (14). Analyses of the available *Brucella* genomes did not uncover an IrrE-like protein. Moreover, the observation that expression of the *lacZ* fusion was low in a *B. abortus recA* mutant and was not induced by MC damage is inconsistent with the notion of a RecA-independent positive regulator. Instead, we present evidence that high basal RecA levels in *B. abortus* are achieved by expressing an activated RecA protein that constitutively cleaves LexA without the typical requirements for inducing treatments.

In the characterizations of *B. abortus* strains carrying the *recA-cat* disruption mutation (and structure confirmed), we were surprised to discover that these strains were not hypersensitive to UV damage, as has been seen for most bacteria carrying *recA* null mutants (17, 20, 46). As documented for *M. xanthus* (42) and for *B. megaterium* (38), the expression of a "second" RecA homologue in *B. abortus* was considered. We reasoned that a "second" *recA* could partially compensate for the inactivation of the "first" *recA* gene, but efforts to detect or clone this postulated "second" *recA* in *B. abortus* were unsuccessful. Moreover, after the *Brucella* genomes became available, BLASTP searches uncovered only one gene encoding RecA for each of these pathovars. However, the next closest match in the *Brucella* spp. was a gene encoding the RadA protein, a highly diverged and poorly characterized DNA repair protein. The *radA* gene was initially identified in *E. coli* as a mutant cell that was mildly sensitive to a number of mutagenic agents, including gamma radiation, MMS, and UV radiation (4, 12, 41, 49, 53).

We investigated the possibility that the *B. abortus* RadA might act as a functional homologue of RecA and thus compensate for the loss of the RecA protein. We first amplified and cloned the *B. abortus radA* gene and then demonstrated that this gene could complement a *radA* defect in *E. coli*. We next constructed a null allele of the *B. abortus radA* gene, and this mutant strain conferred a modest DNA repair-sensitive phenotype, similar to *radA* mutants of *E. coli* (49). To investigate a possible functional overlap between RecA and RadA in *Brucella*, we constructed a *recA radA* double mutant of *B. abortus*. The UV sensitivity phenotypes for the double mutant were essentially the same as that of the *recA* mutant. Although RadA may have some role in DNA repair, we conclude that its role is relatively minor compared to RecA and does not act in place of RecA.

Since bacteria engulfed by phagocytes are known to suffer severe DNA damage from the intracellular burst (51), we investigated whether DNA repair mutants became more sensi-

tive within macrophages. The *B. abortus recA* mutant exhibited a nearly fourfold decline in survival to murine peritoneal macrophages but nominal sensitivity for the *uvrA* and *radA* repair mutants (Fig. 7 and data not shown). The respiratory burst of ROI and RNI in macrophages is enhanced by pretreatment with IFN-γ and corresponds with increased killing of *B. abortus* (24–26). IFN-γ activation exacerbated the sensitivity for the *recA* mutant but had little, if any, effect on the other two repair mutants examined (data not shown). The increased sensitivity indicates that the repair activities of RecA, such as homologous recombination and/or elevated expression of the SOS regulon, play an important role in surviving both ROI and RNI DNA damage within macrophages. We noted that the *recA* mutant was slightly sensitive to $H_2O_2$ using a plate assay. The sensitivity of the *B. abortus recA* mutant may be formally similar to that observed in earlier studies where *E. coli* and *S. enterica* serovar Typhimurium *recA* mutants were found to be sensitive to $H_2O_2$ and activated macrophages (2, 7, 8, 21, 29). Hydrogen peroxide induces strand breaks and a number of covalent modifications to DNA, which contributes to the induction of the SOS response and utilizes a number of response systems, including recombinational repair (20, 21, 29). The modest sensitivity of the *Brucella recA* mutant to $H_2O_2$ (Fig. 6) could indicate that much of the intracellular killing is the result of other substances released in macrophages. The RNI that are also released in the oxidative burst of macrophages are known to be potent mutagens, producing damaged bases, abasic lesions, and single- and double-stranded DNA breaks; the former are removed by adaptive response, whereas the breaks are repaired by recombinational repair (20, 25, 36, 54, 55). We speculate that an important role for RecA may be recombinational repair of ROI- and/or RNI-induced DNA breaks in macrophages. We suggest that this sensitivity of the *B. abortus recA* mutant in activated macrophages reflects two requirements for RecA: first, its direct role in promoting homologous recombination, and second, a regulatory role in assuring high expression levels of SOS repair functions.

Although we show that *recA* mutants of *B. abortus* are hypersensitive to macrophages, RecA does not appear to be required to persist in mice (56). Despite approximately 100-fold-lower splenic counts of the *recA* mutants, the bacteria nonetheless persisted for months after infection of the animals. We suspect that this apparent difference in macrophages and in vivo may reflect requirements for RecA in surviving the initial respiratory burst of macrophages but may be less important during the prolonged intracellular survival after the initial burst. An alternate explanation for persistence of a *recA* mutant in vivo is to postulate the repair activity of another unknown protein(s) that compensates for RecA, presumably a functional homologue that has little sequence identity with RecA. Recent studies with *E. coli* may offer some insights. For instance, the *E. coli* MgsA protein, with DNA-dependent ATPase and single-stranded DNA annealing activities, has recently been described to have overlapping functions with RecA (52). It would be interesting to determine if a hyperactive MgsA-like protein in *B. abortus* might compensate for RecA. Another explanation would be that the damage is tolerated in a RecA-independent pathway; there is evidence for some low levels of UV-induced recombination in *E. coli* that are RecA independent (43). In efforts to elucidate this *recA*-independent UV repair process of *Brucella*, we have initiated genetic approaches to identify the gene(s) that codes for this repair function(s). One approach we are employing is an open-ended genetic screen to isolate mutants of the *B. abortus recA*::*cat* strain that are hypersensitive to UV.

In conclusion, we have found that the *B. abortus* SOS regulatory networks share a number of similar traits with other bacteria that also survive in extreme environments, including high basal *recA* expression and low induction upon DNA damage. Unlike *B. megaterium*, the modest sensitivity to UV radiation for the *B. abortus recA* mutant was not the result of an auxiliary *recA* gene. We presume that some unknown function in *Brucella* is acting in the place of RecA to repair UV damage. But RadA with the greatest sequence identity to RecA did not have overlapping functions and could not account for the modest sensitivity to UV. The mechanism(s) and the identification of the function(s) that promotes this RecA-independent UV repair in *B. abortus* are the subject of intense study.

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