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Acinetobacter baumannii RecA Protein in Repair of DNA Damage, Antimicrobial Resistance, General Stress Response, and Virulence[▽]

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RecA is the major enzyme involved in homologous recombination and plays a central role in SOS mutagenesis. In *Acinetobacter* spp., including *Acinetobacter baumannii*, a multidrug-resistant bacterium responsible for nosocomial infections worldwide, DNA repair responses differ in many ways from those of other bacterial species. In this work, the function of *A. baumannii* RecA was examined by constructing a *recA* mutant. Alteration of this single gene had a pleiotropic effect, showing the involvement of RecA in DNA damage repair and consequently in cellular protection against stresses induced by DNA damaging agents, several classes of antibiotics, and oxidative agents. In addition, the absence of RecA decreased survival in response to both heat shock and desiccation. Virulence assays *in vitro* (with macrophages) and *in vivo* (using a mouse model) similarly implicated RecA in the pathogenicity of *A. baumannii*. Thus, the data strongly suggest a protective role for RecA in the bacterium and indicate that inactivation of the protein can contribute to a combined therapeutic approach to controlling *A. baumannii* infections.

After DNA damage, the gaps produced during the replication process are repaired by enzymes of the recombination system. RecA is the major enzyme involved in homologous genetic recombination and recombinational repair. The protein coats single-stranded DNA (ssDNA) and causes it to invade double-stranded DNA (dsDNA), thereby catalyzing strand exchange between DNA molecules (37). In addition, RecA is central to the repair response known as SOS mutagenesis (11). The interaction of RecA with ssDNA (resulting from DNA damage) leads to autocatalysis of LexA, which normally inhibits the SOS response (26). While several SOS response enzymes appear to be highly error prone, they nonetheless allow the replication fork to proceed over damaged DNA, guaranteeing DNA replication and thus cell survival (45).

The Gram-negative coccobacillus *Acinetobacter baumannii is* responsible for nosocomial infections worldwide, especially in intensive care units (32, 33). This multidrug-resistant pathogen can survive for months on surfaces, which thereby act as a continuous source of transmission if preventive disinfection is inadequate (25). In *Acinetobacter baylyi* ADP1, SOS mutagenesis does not occur in response to DNA damage (39), and in *Acinetobacter* spp. no LexA homolog has been identified (20, 38), implying that DNA repair proceeds by other mechanisms. This observation, together with the well-established involvement of RecA in both the stress response and bacterial virulence, led us to further study the functions of this protein. Specifically, we constructed an *A. baumannii recA* mutant in order to examine the role of RecA in the repair of DNA damage, antibiotic resistance, the general stress response, and virulence.

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MATERIALS AND METHODS

Growth conditions and antibiotic susceptibilities. *Escherichia coli* TG1 (used for cloning) and *A. baumannii* (ATCC 17978 and derivative strains) were grown in Mueller Hinton (MH) medium (Difco). When necessary, kanamycin (50 μg/ml), rifampin (50 μg/ml), and zeocin (200 μg/ml) were added to the growth media. All cultures were incubated at 37°C with shaking at 180 rpm. The MICs of the tested antibiotics were determined by Etest (AB Biodisk) following the manufacturer's instructions.

Mutant construction. An *A. baumannii recA* mutant was obtained by insertional mutagenesis using allelic replacement. Briefly, the *recA* gene of *A. baumannii* strain ATTC 17978 was PCR amplified using the primers EnRecAab-up and EnRecA-ab-rv (Table 1), and the amplicon was cloned into the pGEM-T vector (Promega). The entire construct was then amplified by overlap-extension PCR using *Pwo* DNA polymerase (Roche), which generates blunt-ended PCR products, and primers RecABI-up and RecABI-rv (Table 1), which anneal to an internal region of *recA*. The kanamycin (Km) cassette was PCR amplified using the *Pwo* DNA polymerase, the Kmup and Kmrv primers (Table 1), and the pCR-BluntII-TOPO plasmid (Invitrogen) as a template and then ligated into the entire construct using T4 DNA ligase (Roche). The resulting *recA*::Km fragment was PCR amplified with the primers EnRecAab-up and EnRecA-ab-rv, and the resulting PCR product was electroporated into *A. baumannii* strain ATCC 17978. Allelic replacement was carried out as described previously (2). Gene replacement in candidate clones was confirmed by PCR and by sequencing (Macrogen Sequencing Service) of the region in the resulting mutant using primers RecAext-up and RecAext-rv (Table 1).

Complementation. The *A. baumannii recA* mutant was complemented as follows: the *recA* open reading frame (ORF) was amplified with the RecA-XbaI and RecA-NcoI primers (Table 1) from the genome of *A. baumannii* strain ATCC 17978 and cloned into the XbaI-NcoI restriction site of the pET-RA vector under the control of the β-lactamase CXT-M-14 gene promoter (2), yielding the pET-RA-RecA plasmid. The *A. baumannii recA* mutant was transformed with pET-RA and the recombinant pET-RA-RecA plasmid. Transformants were selected on rifampin- and kanamycin-containing plates and confirmed by PCR using the pETRAFW and pETRARV primers (Table 1).

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TABLE 1. Oligonucleotides used in this work

Oligonucleotide	Sequence (5′ to 3′)[a]	Application
EnRecAab-up	TAGGTCAGCTTACAGAGC	Mutant construction
EnRecAab-rv	GTAGCAGATCTATGATGTGC	Mutant construction
RecABI-up	CTACCACTGGTGGTAACG	Mutant construction
RecABI-rv	GTGCCTGGCTCATAAGAC	Mutant construction
Kmup	TGGACAGCAAGCGAACCG	Mutant construction
Kmrv	AGAACTCGTCAAGAAGGC	Mutant construction
RecAext-up	CCAATTCTGGAAAGAGTTGC	Mutant verification
RecAext-rv	GCTGAGGAATTAATCAATCG	Mutant verification
RecA-XbaI	ATCGTCTAGAATGGATGAG AATAAAAGC	Complementation
RecA-NcoI	ATCGCCATGGTTACGATTCT AATAAAAG	Complementation
pETRAFW	TTCTTCGTGAAATAGTG ATGATT	Cloning verification
pETRARV	CTGTTTCATATGATCTGG GTATC	Cloning verification

[a] Restriction endonuclease recognition sites are underlined.

UV resistance assays. Strains in the exponential growth phase (optical density at 600 nm [OD_{600}] of 0.5) were washed and suspended in NaCl (0.9%) and then thinly spread on a sterile petri plate. The plates were then irradiated with UV-C light (254 nm) at a dose of 0 to 30 J/m^2 using a Bio-Link BLX-254 cross-linker (Vilber Lourmat). For each UV-C dose, samples were removed, diluted, and plated in MH medium. All experiments with UV-irradiated cells were performed in the dark to prevent photoreactivation of pyrimidine dimers. The plates were immediately wrapped in aluminum foil and incubated at 37°C for 24 h. The survival percentage was calculated as the number of CFU at each dose divided by the total number of CFU at a dose of 0 J/m^2.

Recombination assays. The suicide vector pTOPO33int (2), carrying a 387-bp fragment of the *A. baumannii* ATCC 17978 genome and a zeocin gene resistance, was electroporated in the corresponding *A. baumannii* strains as previously described (2). Recombinants were selected on zeocin-containing plates. Frequency of recombination was calculated as the number of recombinants obtained divided by the total number of CFU.

Oxidative damage assays. Strains in the stationary growth phase (OD_{600} of 1.4) were incubated in MH broth with hydrogen peroxide (H_2O_2) added to a final concentration of 30 mM (46). The cultures were incubated for 30 min at 37°C with shaking and then immediately serially diluted and plated onto MH plates. The survival percentage was calculated as the number of CFU divided by the total number of CFU (bacteria that did not undergo H_2O_2 treatment).

Disc diffusion assays. Susceptibility to menadione, sodium nitroprusside, hydrogen peroxide, mitomycin C, and ethidium bromide was tested by the disc diffusion method. Plates were inoculated by dipping sterile cotton swabs into the standard inoculum suspension (approximately 10^6 CFU/ml) and evenly streaking the entire surface. Filter discs were placed on the plates and spotted with aliquots (10 μl) of 1 M menadione, 5 M sodium nitroprusside, 1 M hydrogen peroxide, mitomycin C (0.5 mg/ml), or ethidium bromide (5 mg/ml). The diameters of the resulting growth inhibition areas were measured after incubation at 37°C for 24 h.

Heat shock resistance assays. Strains in the exponential growth phase (OD_{600} of 0.5) were placed in culture tubes and incubated at 55°C. Samples (0.1 ml) withdrawn before (time zero) and 20 and 40 min after heat exposure were then diluted and plated. The resulting colonies were counted after 24 h of incubation at 37°C. Percent survival was calculated as the number of CFU at each time point divided by the number of CFU at time zero.

Desiccation resistance assays. Dilution series of exponentially growing cells (OD_{600} of 0.5) were spotted on sterile cellulose filters (0.45-μm pore size; Millipore). The filters were either not dried (control) or dried for 3, 6, or 24 h inside a sterile petri plate at 37°C before they were placed on tryptic soy agar (TSA) plates and incubated for 24 h at 37°C.

***In vitro* assay for *A. baumannii* survival within macrophages.** Cells (5×10^5) from the mouse leukemic monocyte macrophage cell line Raw 264.7 were allowed to adhere to the wells of a 24-well plate, after which the bacteria were added at a ratio of 10 to 20 bacteria per macrophage. The plates were centrifuged at 250 × *g* for 5 min at room temperature to enhance and synchronize infection and then incubated for 1 h at 37°C to permit phagocytosis. Free bacteria were subsequently removed by three washes with 0.9% NaCl. Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml) was added, and the cells were then incubated again at 37°C. Wells were sampled at 0 and 1 h by aspirating the medium, lysing the macrophages with 0.5 ml of 0.5% deoxycholate, rinsing each well with 1 ml of NaCl 0.9%, and plating 0.1 ml of the lysate onto MH plates. A minimum of four wells were plated for each bacterial strain at each time. The survival percentage was calculated as the number of CFU at 1 h divided by the number of CFU at 0 h.

Virulence assay. ICR (CD1) female mice (Harlan Interfauna Ibérica, Barcelona, Spain) weighing 25 g each were acclimated to standard laboratory conditions with a 12-h light/12-h dark cycle. Antibiotic-free pelleted food and autoclaved water were provided *ad libitum* during the assay. All experiments involving mice were approved by the Animal Ethics Committee of our institution. The mice were intraperitoneally injected with a 0.1-ml volume of the bacterial suspension. The concentrations of the original bacterial suspensions were determined by the plate count method. A series of preliminary and independent trials were performed for both the wild-type (WT) strain ATCC 17978 and the *A. baumannii recA* mutant (from 5×10^6 to 3.5×10^8 CFU/mouse) to establish the optimal bacterial dose for the virulence assay. Preliminary results from these trials were consistent with those obtained in the main experiment, conducted as follows. Two groups of 15 mice each were intraperitoneally inoculated with approximately 2×10^8 CFU of either the WT or the *recA A. baumannii* strain/mouse and monitored every 12 h for mortality during 7 days postinoculation.

Statistics. Unless otherwise specified, all data are expressed as means ± standard deviations and were analyzed by two-tailed, one-way analysis of variance (ANOVA), followed by the Tukey test for posthoc multiple group comparisons. All assays (except those involving mice) were repeated at least three times. In all cases, a *P* value of <0.05 was considered statistically significant.

RESULTS

Phenotypic characterization of the *A. baumannii recA* mutant. The growth rate of the *A. baumannii recA* mutant was similar to that of the parent strain (Fig. 1). Since *recA* mutants are expected to be significantly more sensitive to DNA damaging agents than their isogenic parental strain (24), the *A. baumannii recA* mutant was tested for its sensitivity to mitomycin C, ethidium bromide, and UV irradiation. Susceptibility to mitomycin C (a potent cross-linker) and ethidium bromide (an intercalating agent) was tested by the disc diffusion method, measuring the diameters of the growth inhibition areas. The results showed that the *recA* mutant and the control strain carrying the pET-RA plasmid without an inserted *recA*

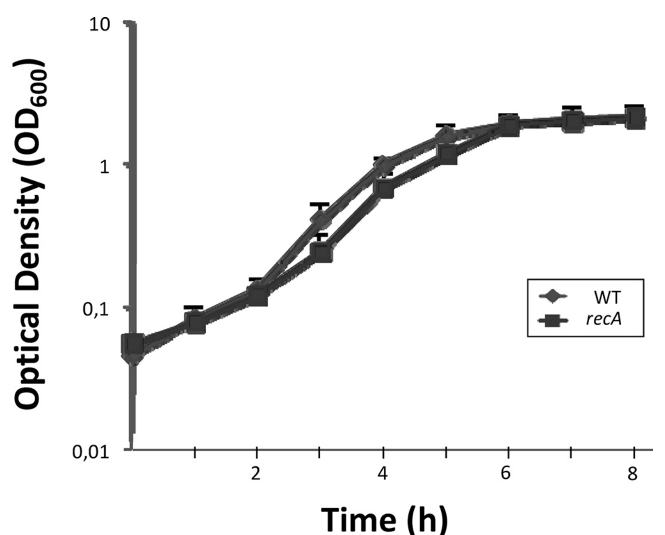

FIG. 1. Growth curves of *A. baumannii* WT and *recA* mutant strains in MH medium. Error bars represent the standard error of the mean of three independent experiments. Significance was determined at a *P* value of >0.05 according to a paired, two-tailed Student's *t* test.

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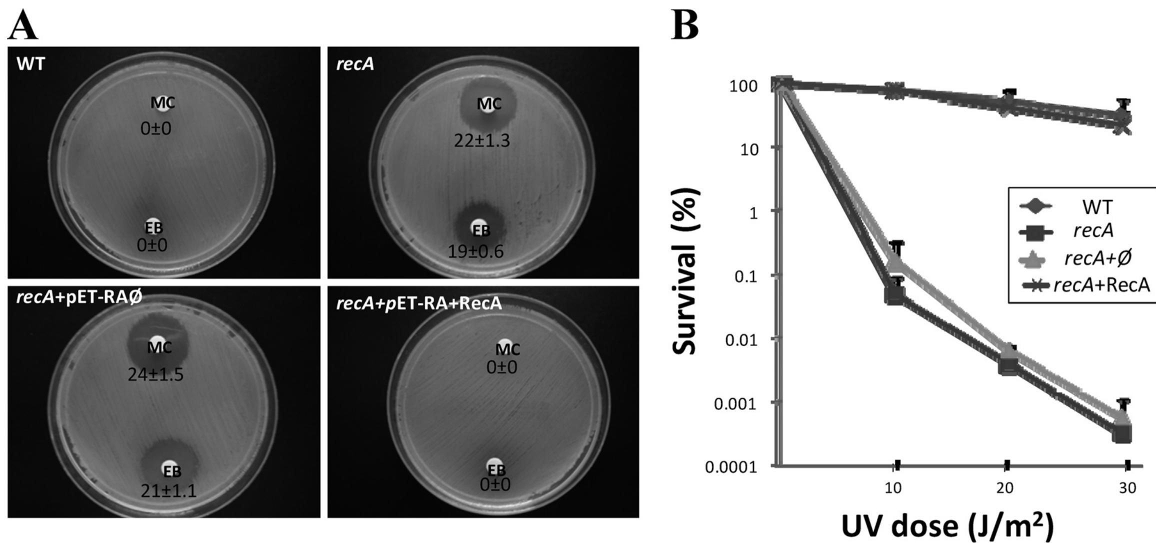

FIG. 2. (A) Ethidium bromide (EB) and mitomycin C (MC) resistance of the ATCC 17978 parent strain (WT), the *recA* mutant (*recA*), the *recA* mutant carrying the empty pET-RA plasmid (*recA*+pET-RAØ), and the complemented *recA* mutant (*recA*+pET-RA+RecA) as determined by disc diffusion assays. The means ± standard deviations of the inhibition halos from three independent experiments are indicated in mm for each strain. Significance was determined at a *P* value of <0.01 in comparisons of the WT versus the *recA* mutant and of the *recA* mutant with the empty pET-RA plasmid versus the complemented *recA* strain for both EB and MC. (B) *A. baumannii* survival after exposure to UV light. Cultures were UV irradiated, and the percentage of surviving cells was determined by comparison with nonirradiated cells. Error bars represent the standard error of the mean of three independent experiments. Significance was determined at a *P* value of <0.01 in comparisons of WT versus the *recA* mutant and of the *recA* mutant carrying the empty pET-RA plasmid versus the complemented *recA* strain.

gene were significantly more sensitive ($P < 0.01$) to both agents than the WT and complemented strain, respectively (Fig. 2A). A UV survival curve constructed for the same strains showed that the *recA* mutant's resistance to UV exposure was significantly less ($P < 0.01$) than that of the parental strain (Fig. 2B). At each UV light dose tested, the *recA* mutant was at least 1,000-fold more sensitive to UV irradiation than the WT parent, indicating the involvement of *A. baumannii* RecA in the repair of UV-induced damage. Furthermore, as expected, the mutant complemented strain completely restored the WT phenotype, whereas the negative control carrying the same plasmid without *recA* did not (Fig. 2B). This increased sensitivity of the *recA* mutant to DNA damaging agents is consistent with a *recA* mutant phenotype (24, 27). In addition, homologous recombination was not detected in the *recA* mutant (Table 2).

The *A. baumannii recA* mutant is more sensitive to oxidative stress than the WT parent. Oxidative damage to DNA is mediated by reactive oxygen species (ROS), in particular, the hydroxyl radical (4), which is generated from the reaction of normally DNA-inert hydrogen peroxide and either Fe^{2+} or Cu^{+} through the Fenton reaction (8). Therefore, the *in vitro* ability of the *recA* mutant to survive H_2O_2 challenge was evaluated (Fig. 3A). The results showed significant survival differences ($P < 0.01$) between the *recA* mutant (3.8%) and the WT (47.2%) strains. In fact, the mutant was approximately 10-fold more sensitive than its WT parent to the lethality of H_2O_2.

In the presence of superoxide anion radicals (O_2^-), the reactive nitrogen intermediate (RNI) nitric oxide (NO) forms the stable peroxynitrite anion ($ONOO^-$), which, like other chemical oxidants, damages DNA (28). To further confirm the sensitivity of the *A. baumannii recA* mutant to oxidative agents, its response to sodium nitroprusside (NO donor), H_2O_2, and menadione (an O_2^- donor that can be dismutated into H_2O_2) was tested by the disc diffusion method (Fig. 3B). The results clearly showed that the *recA* mutant's sensitivity to all three oxidative agents was much greater than that of the WT parent ($P < 0.01$ in all cases) (Fig. 3B).

Effect of RecA on antibiotic resistance in *A. baumannii*. To determine whether a lack of RecA alters antimicrobial suscep-

TABLE 2. Recombination abilities of the indicated *A. baumannii* strains

ATCC 17978 genotype[a]	No. of recombinants[b]				Total no. of viable CFU/ml	Frequency of recombination
	Expt 1	Expt 2	Expt 3	Control		
WT	224	218	134	0	1×10^8	1.92×10^{-5}
recA	0	0	0	0	1.8×10^8	$<1.8 \times 10^{-8}$
recA pET-RAØ	0	0	0	0	2.5×10^8	$<2.5 \times 10^{-8}$
recA pET-RA-RecA	18	26	31	0	1.4×10^8	1.79×10^{-6}

[a] pET-RAØ, empty pET-RA vector.
[b] Number of recombinants obtained in three independent electroporations of 0.1 ml of the indicated *A. baumannii* strains with (Expt 1 to 3) or without (control) 0.1 μg of pTOPO33int plasmid.

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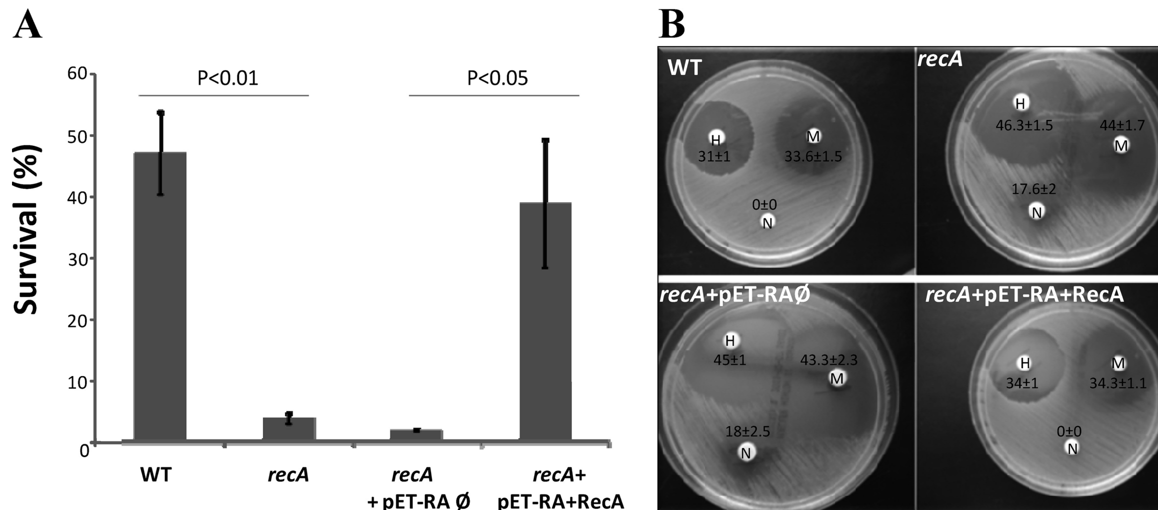

FIG. 3. (A) Hydrogen peroxide resistance of the indicated strains of *A. baumannii*. Bacterial cells were treated with 30 mM H_2O_2 for 30 min. Error bars represent the standard error of the mean of three independent experiments. Significance was determined at *P* values of <0.01 and <0.05 in comparisons of the WT versus the *recA* mutant and of the *recA* mutant carrying the empty pET-RA plasmid (*recA*+pET-RAØ) versus the complemented *recA* mutant (*recA*+pET-RA+RecA), respectively. (B) Hydrogen peroxide (H), menadione (M), and sodium nitroprusside (N) resistance of the indicated strains as determined by disc diffusion assays. The means ± standard deviations of the inhibition halos from three independent experiments are indicated in mm for each compound in each strain. Significance was determined at a *P* value of <0.01 in comparisons of the WT versus the *recA* mutant and of the *recA* mutant carrying the empty pET-RA plasmid versus the complemented *recA* mutant in all cases.

tibilities, the *recA* mutant's response to antibiotics of different classes was tested (Table 3). Compared to the WT parent strain, the *A. baumannii recA* mutant had a 2- to 4-fold higher susceptibility to β-lactams (piperacillin, piperacillin-tazobactam, amoxicillin-clavulanic acid, imipenem, ceftazidime, and cefotaxime), colistin, and trimethoprim-sulfamethoxazole (Table 3). The highest susceptibilities (approximately 15- to 30-fold) occurred in response to quinolone-type antibiotics (Table 3). In contrast, the mutant and the WT parent did not differ significantly in their susceptibilities to rifampin and antibiotics that act by inhibiting protein synthesis, such as macrolides (erythromycin), aminoglycosides (tobramycin and amikacin), and chloramphenicol (Table 3).

TABLE 3. MICs of the antimicrobials used in this study for the indicated *A. baumannii* strains

Antibiotic	MIC of the indicated ATCC 17978 strain(μg/ml)[a]			
	Parent strain	*recA* mutant	*recA* pET-RAØ strain[b]	*recA* pET-RA-RecA strain
Piperacillin	32	12	12	24
Piperacillin/tazobactam	16	6	6	12
Amoxicillin/clavulanic acid	64	24	24	48
Imipenem	0.38	0.19	0.19	0.38
Ceftazidime	6	3	3	6
Cefotaxime	96	48	48	128
Colistin	0.5	0.25	0.25	0.38
Trimethoprim/sulfamethoxazole	4	0.75	0.75	6
Ciprofloxacin	0.25	0.016	0.016	0.25
Moxifloxacin	0.094	0.003	0.003	0.094
Erythromycin	4	4	4	6
Chloramphenicol	>256	>256	>256	>256
Tobramycin	0.38	0.38	0.38	0.38
Amikacin	1.5	1.5	1.5	2
Rifampin	4	4	NA	NA

[a] NA, not applicable (the pET-RA plasmid carries rifampin resistance).
[b] pET-RAØ, empty pET-RA vector.

The *A. baumannii recA* mutant is more sensitive to heat shock and desiccation than the WT parent. Heat stress can induce DNA damage through replication fork stalling (16). To assess heat tolerance by the *recA* mutant, its survival after various periods of incubation at 55°C was tested. Over time, the *recA* strain lost viability more rapidly than either the WT or the complemented strain (Fig. 4). After 40 min of exposure to the elevated temperature, the number of surviving *recA* mutant cells was 10-fold lower than that of the WT or complemented strain (Fig. 4), demonstrating the importance of RecA in heat shock resistance.

Desiccation is inherently DNA damaging, and the DNA of dried bacterial cells exhibits a substantial number of double- and single-stranded breaks as well as cross-links (14). Accordingly, the resistance levels of the *recA* mutant, WT, and complemented strains under drought conditions were compared (Fig. 5). Aliquots of the strains were dried on filters for 3, 6, or 24 h inside a sterile petri plate at 37°C, after which the ability of the bacteria to form colonies on agar plates was determined. In the control experiment, the filters were placed directly on the agar plates and then incubated. No difference between the strains was observed on nonexposed filters, whereas in the desiccation experiments the viability of the *recA* mutant dramatically decreases (Fig. 5). Furthermore, as expected, the mutant complemented strain completely restored the WT phenotype, whereas the negative control carrying the same plasmid without *recA* did not (Fig. 5).

Survival of an *A. baumannii recA* mutant within macrophages. As part of the host response to infection, macrophages generate an oxidative burst that produces oxidizing compounds able to damage DNA (41). To determine whether the *A. bau-*

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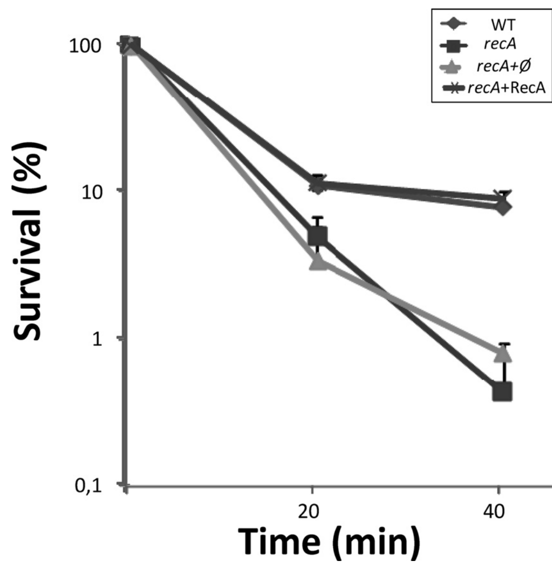

FIG. 4. Thermal resistance of the WT parent strain, the *recA* mutant, the *recA* mutant with the empty pET-RA plasmid (*recA*+Ø), and the complemented *recA* mutant (*recA*+RecA). The viability of cultures directly challenged at 55°C was determined by plating on MH medium at the indicated times. Error bars represent the standard error of the mean of three independent experiments. Significance was determined at a P value of <0.01 in comparisons of the WT versus the *recA* mutant and of the *recA* mutant carrying the empty pET-RA plasmid (*recA*+pET-RAØ) versus the complemented *recA* mutant (*recA*+pET-RA+RecA).

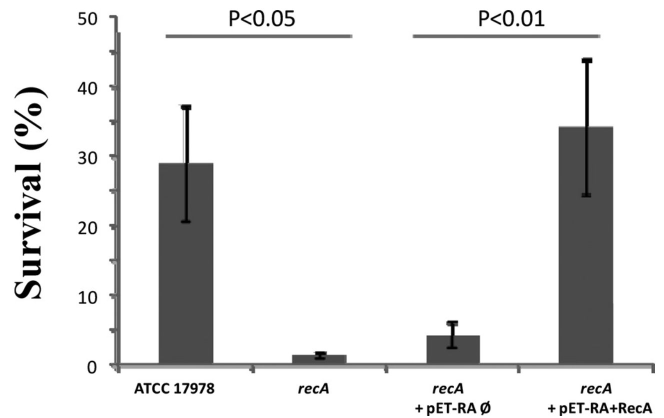

FIG. 6. Survival of the indicated strains of *A. baumannii* after a 1-h incubation with macrophages. Significance was determined at P values of <0.05 and <0.01 in comparisons of WT versus the *recA* mutant and of the *recA* mutant carrying the empty pET-RA plasmid (*recA*+pET-RAØ) versus the complemented *recA* mutant (*recA*+pET-RA+RecA), respectively.

mannii recA mutant was more sensitive to macrophages, its survival in the presence of these cells was compared with that of the WT parent. After a 1-h incubation with 5×10^5 mouse leukemic monocyte macrophages, there were approximately 20-fold fewer viable *recA* mutant than WT cells (Fig. 6). Furthermore, as expected, the mutant complemented strain completely restored the WT phenotype, whereas the negative control carrying the same plasmid without *recA* did not (Fig. 6). These data indicated that the *A. baumannii* RecA protein plays

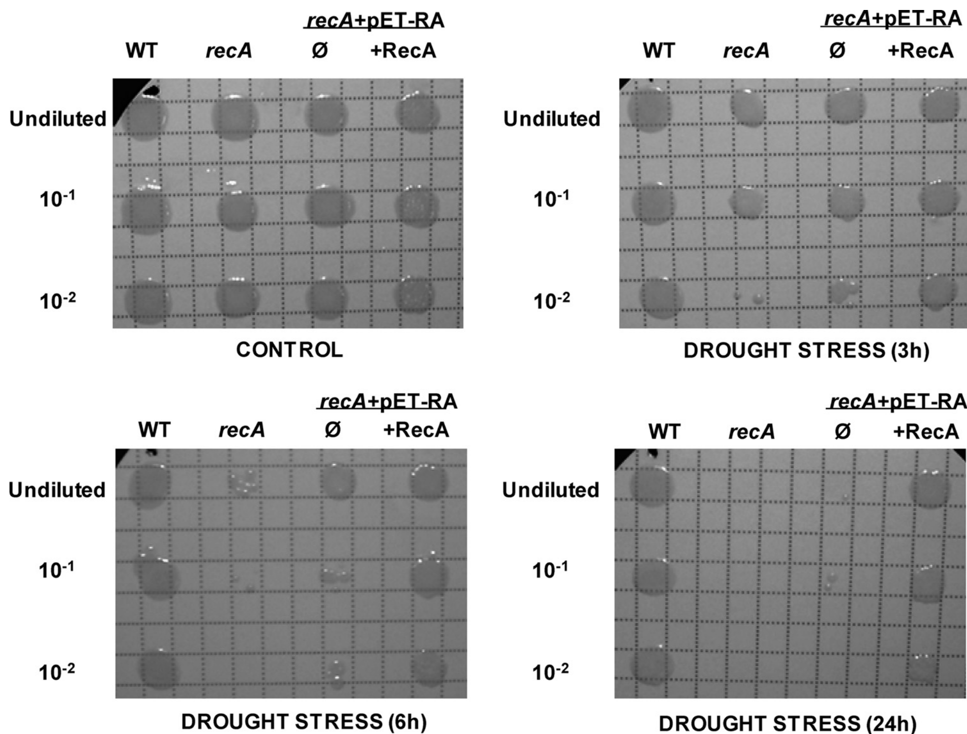

FIG. 5. Desiccation test with the indicated *A. baumannii* strains. Cell viability was tested by spotting dilution series of cells in the exponential growth phase on sterile filters subsequently exposed to TSA plates either immediately (control) or after 3, 6, or 24 h at 37°C (drought stress). *recA*+pET-RA+RecA, complemented *recA* mutant; *recA*+pET-RAØ, *recA* mutant carrying the empty pET-RA plasmid.

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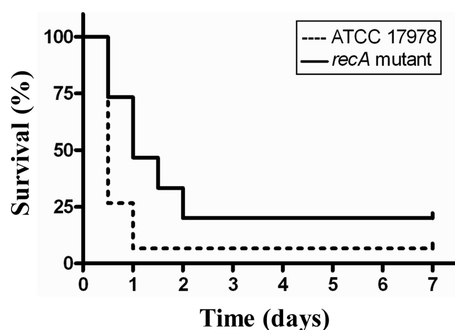

FIG. 7. Survival of mice (n = 15 per group) inoculated with the *A. baumannii* WT or *recA* mutant strain. Significant differences in survival were noted (log rank test, $P < 0.05$).

a significant role in conferring resistance to the lethal products released during the oxidative burst of activated macrophages and were consistent with the *recA* mutant's sensitivity to oxidative and nitrosative damage.

Virulence in a mouse model of systemic infection. To study the effect of *recA* inactivation on virulence *in vivo*, the mortality rates of groups of 15 mice inoculated with either WT or *recA* mutant strain were monitored for a period of 7 days (Fig. 7). During the first 24 h postinoculation, 73.3% of the mice injected with the WT strain died, whereas only 26.7% of the mice injected with the *recA* mutant died. At day 7 postinoculation, 20% of the mice inoculated with the *recA* mutant survived, while only 6.7% of the mice injected with the WT strain survived until the end of the trial. The results clearly show that *recA* inactivation modified the survival curve, implying a significantly reduced virulence of the mutant compared with the WT ATCC 17978 strain ($P < 0.05$, log-rank test). Thus, *recA* expression appears to be involved in the virulence of *A. baumannii*.

DISCUSSION

In *E. coli* and many other bacterial species, SOS mutagenesis is activated in response to DNA damage that is so extensive it cannot be repaired by other pathways (17, 45). In mutagenic repair, several genes are upregulated, including those encoding DNA polymerases, such as the error-prone DNA polymerases IV and V, and SulA, which temporarily causes the arrest of cell division, allowing the replication fork to proceed over the damaged DNA (45). However, *Acinetobacter* spp. lack not only SulA but also the SOS repressor LexA (39), and *A. baylyi* ADP1 does not respond to DNA damage by initiating SOS mutagenesis (39). Nevertheless, our data clearly show that RecA is involved in DNA repair in *A. baumannii* exposed to DNA damaging agents such as ethidium bromide, mitomycin C, and UV irradiation (Fig. 2). Since a typical SOS response seems to be absent in this bacterial species and since homologous recombination is lost in the *recA* mutant (Table 2), these results suggest that while DNA repair is mainly carried out by RecA, the protein functions not as an activator of the SOS response but, rather, through the recombinational repair pathway.

Hydroxyl radicals produce a multiplicity of DNA modifications, including strand breaks, base loss or damage, and fragmentation of the deoxyribose moiety (40). The ability of *recA* to confer protection against oxidative damage has been demonstrated in *E. coli* (1), *Salmonella enterica* serovar Typhimurium (6), *Lactococcus lactis* (16), *Neisseria gonorrhoeae* (49), and *Bacteroides fragilis* (48). In contrast to these species, the *recA* mutant strain, like the parental strain of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (31), is resistant to H_2O_2 killing. *E. coli* RecA is important in the repair of oxidatively damaged DNA, both directly for its functions in DNA repair and indirectly for its role in the induction of the SOS response (21, 23). Our results show that, following the exposure of *A. baumannii* to the oxidative stress produced by ROS and RNI, RecA is needed for bacterial survival (Fig. 3) but probably only through the recombinational repair pathway. Similarly, in *N. gonorrhoeae*, which also lacks a classical SOS response, RecA and DNA recombinational repair enzymes confer resistance to oxidative damage (49).

Some bactericidal antimicrobials stimulate the production of highly deleterious ROS radicals, which contribute to the generation of hydroxyl-radical-mediated DNA damage and, consequently, to cell death (22). Likewise, inactivation of the *recA* gene of *E. coli* is known to increase bacterial susceptibility to several antibiotics (22, 50). We obtained similar results in tests of the susceptibility of the *A. baumannii recA* mutant to different groups of antimicrobials (Table 3). In particular, the highest antibiotic sensitivity occurred in response to treatment with quinolones (Table 3). This result was not surprising since the primary DNA damaging actions of quinolones, the conversion of their DNA gyrase and topoisomerase targets into endonucleases (15), are amplified by ROS (22).

The expression of genes associated with different types of stress is also mediated by *recA* (10). For example, the heat shock response is a highly conserved genetic response to environmental stress and is ubiquitous in nature. Indeed, it has been found in every organism studied thus far, from archaea to mammals (13). We observed that the *A. baumannii recA* mutant is more sensitive to heat shock than the parental strain (Fig. 4). Similarly, *recA* mutants of *E. coli*, *L. lactis*, and *Streptococcus thermophilus* are also more sensitive to heat shock than the respective WT parental strain (13, 19, 42). In contrast, although the *recA* gene of *X. oryzae* pv. *oryzae* is highly induced by treatments with chemical mutagens, UV, and oxidizing agents, the gene is not induced by heat shock (36). Interestingly, RadA proteins, which are homologous to RecA, have been described in *Archaea* such as *Pyrococcus furiosus* (43). In this hyperthermophilic archaeon, RadA is constitutively expressed, probably in order to repair the damage inflicted on the DNA by the constant exposure to high temperatures. Drought stress is another type of DNA damage likely to cause breaks and cross-links. In fact, our results showed that under desiccation stress, the viability of the *A. baumannii recA* mutant dramatically decreases (Fig. 5). Similarly, sensitivity to drying is higher in *E. coli*, *Mycobacterium smegmatis*, and spores of *Bacillus subtilis* carrying an inactivated *recA* gene than in the respective parent strains (3, 34, 44).

In *Staphylococcus aureus* (5) and enteropathogenic *E. coli* (30), the SOS response induces the synthesis of virulence factors. It also promotes autocleavage of phage repressors, leading to the horizontal spread of temperate phage and associated pathogenicity islands, such as those containing genes encoding

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S. aureus virulence factors (51), the *E. coli* Shiga toxin (53), and the *Vibrio cholerae* toxin as well as antibiotic resistance genes (35). Given the dual role of RecA (in recombination and as an activator of the SOS response), the decreased virulence of *recA* mutants may be attributed to a reduction in recombination ability, inhibition of the SOS system, or both (29). For instance, in *Burkholderia* spp., *recA* mutants are more sensitive than the parent strain to DNA damaging agents, and *recA* inactivation results in an attenuation of virulence (12, 52). In contrast, *recA* inactivation in *Porphyromonas gingivalis* does not alter virulence, as shown in a mouse model, despite the confirmed importance of *recA* in DNA repair in this species (18). Another example of diminished virulence in a *recA* mutant is *Pasteurella multocida*, which is also more sensitive to DNA damaging agents. However, in that species, attenuation is a consequence of the long lag phase of growth rather than a direct effect of *recA* inactivation (9). In *Brucella abortus*, a *recA* mutant showed only modest sensitivity to DNA damage, which was ultimately ascribed to the presence of a second RecA protein (RadA); nonetheless, RecA but not RadA was shown to be required for the bacterium's survival within macrophages (41).

While RecA is thus implicated in the virulence of a wide range of bacterial species, essentially nothing is known about its role in the virulence of *Acinetobacter* spp. This led us to study the relationship between RecA and virulence in the multidrug-resistant *A. baumannii*, which causes a wide spectrum of nosocomial infections. Our *in vitro* model of virulence revealed that the *A. baumannii recA* mutant is approximately 20-fold more sensitive to macrophages than the WT parent strain (Fig. 6). ROS and RNI compounds are excreted at high concentrations by macrophages as part of their microbicidal activities, causing DNA damage during infection (47). This damage no doubt includes single- as well as double-stranded breaks in addition to altered or missing bases (7). Since in *A. baumannii* RecA is directly involved in DNA repair, inactivation of the protein renders this bacterium more sensitive to the oxidative burst of macrophages. In addition, *in vivo* virulence assays in the mouse model showed that, compared with the WT parental strain, the virulence of the *A. baumannii recA* mutant is significantly attenuated (Fig. 7), probably as a result of diminished survival in the host. Similarly, in *S. enterica* serovar Typhimurium, *recA* mutants (which also are more sensitive to DNA damaging agents than the parental strain) are avirulent and more sensitive to the oxidative burst of macrophages (7).

In conclusion, our findings consistently demonstrate that RecA protects *A. baumannii* from DNA damaging agents (i.e., UV, mitomycin C, and ethidium bromide), several classes of antibiotics, chemical oxidants, and other stress factors, such as heat shock and desiccation. In addition, we were able to show, both *in vitro* and *in vivo*, that RecA plays an important role in the pathogenicity of this multidrug-resistant microorganism. Taken together, our observations point to RecA as an attractive target for further studies of *Acinetobacter* virulence. Furthermore, RecA inhibition may be an effective therapeutic strategy in the treatment of *A. baumannii* infections.

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