RecA influences biofilm development in the opportunistic pathogen *Acinetobacter baumannii*

Carly Ching[1,2], Margaret Downs[1,3], Paul Muller[1], Breanna Isley[1], William Fowle[1], and Veronica G. Godoy[1*]

[1]Northeastern University, Department of Biology, Boston, MA, 02115

[2]Current address: Department of Biomedical Engineering, Boston University, Boston, MA, 02215

[3]Current address: Program of Biomedical Sciences, Boston University School of Medicine, Boston, MA 02118.

***** Corresponding Author: Veronica G. Godoy, v.godoycarter@northeastern.edu

Abstract

Acinetobacter baumannii, a Gram-negative opportunistic pathogen, is a leading cause of hospital-acquired infections. *A. baumannii* is difficult to eradicate from hospitals due to its propensity to quickly gain antibiotic resistances and ability to robustly survive on dry surfaces. These strategies are largely mediated by mutagenesis and biofilm development, respectively. Mutagenesis is partly governed by the DNA damage response (DDR). Biofilms are multicellular communities, often surface-attached, that are more difficult to eradicate than free-living planktonic cells. There is increasing evidence that the DDR and biofilm development are linked processes. Here, we show that upon DNA damage, the relative intracellular concentration of RecA, the key DDR protein, is lower than those of *Escherichia coli*. Notably, we report that RecA negatively influences biofilm development. Cells lacking RecA (Δ*recA*), that are unable to upregulate the DDR, have increased surface attachment and sugar content within the biofilm matrix. We further show that in *A. baumannii*, a modest increase in RecA concentrations, akin to DDR induction, decreases surface-attachment. Importantly, biofilms formed by Δ*recA* cells are more difficult to eradicate with

antibiotic treatment. The evidence suggests that the *A. baumannii* DDR influences survival independent from mutagenesis. It also demonstrates the importance of understanding fundamental biology to better appreciate the relationships between different bacterial survival strategies.

Introduction

Acinetobacter baumannii is an emerging Gram-negative opportunistic pathogen and one of the ESKAPE pathogens, a group of bacteria responsible for the majority of hospital-acquired infections [1]. Interest in these bacteria has stemmed from *A. baumannii* outbreaks in hospitals worldwide that are difficult to eradicate, due to increased multi-drug resistance (MDR) [2] and resistance to desiccation [3]. *A. baumannii* is very dangerous to immunocompromised individuals, causing different illnesses, including pneumonia, septicemia, and wound infections [4].

One response pathway that underlies MDR is the DNA damage response (DDR). When gene products involved in antibiotic binding or processing are mutated, resistance is acquired [5]. Mutagenesis can result from induction of error-prone DNA polymerase genes which are part of the DDR regulon, a strategy used by different bacteria [6,7]. In *Escherichia coli* and many other bacteria, the cells' main recombinase, RecA, and the global transcriptional repressor, LexA, manage the DDR, also known as the SOS response [8]. In *A. baumannii*, however, there is no known LexA homologue, making the *A. baumannii* DDR circuitry unique [9,10]. We have shown that in *A. baumannii* expression of multiple error-prone polymerases and clinically-relevant antibiotic resistance acquisition are dependent on RecA [11].

A. baumannii is notorious for its ability to robustly survive on surfaces. Under dry conditions, *A. baumannii* cells have been shown to survive for at least 4 months [3]. Cells that survive on surfaces are often in biofilms, a multicellular community of bacteria embedded in an extracellular matrix of proteins, sugars and/or DNA [12]. Importantly, biofilm-forming clinical *A. baumannii* isolates survived

over two times longer on dry surfaces than isolates that do not form biofilm [13]. This is very dangerous in the clinical setting, where surfaces and equipment provide an opportunity for contamination and spread of *A. baumannii* to immunocompromised individuals.

Bacteria within a biofilm are often less sensitive to chemical and physical challenges, including antibiotics [14,15]**.** Like many biological processes in *A. baumannii*, biofilm development is not fully understood, though certain key biofilm genes have been described, including the adhesive *csu* pili, *bfmR* the biofilm master regulator, Bap protein, and efflux pumps [16–20]. Remarkably, though BfmR is known to play a role in biofilm formation and desiccation tolerance, the regulation of expression of *bfmR* is unknown. BfmR is a response regulator in a two-component system that has been proposed as a drug target specific for *A. baumannii* [20–22]. The *A. baumannii* biofilm extracellular matrix has been reported to contain poly-ß-(1-6)-*N*-Acetyl-Glucosamine, mannose and extracellular DNA [23–26].

In many bacteria, antibiotics and DNA damaging agents often induce biofilm development [27,28]. However, the relationship between the DDR and biofilm development varies between bacterial species. For example, in *Streptococcus mutans*, and *Escherichia coli*, a non-inducible DDR leads to lower biofilm formation [29,30], while in *Clostridium difficile*, a constitutive DDR shows increased biofilm formation [31]. These results suggest a direct correlation between DDR and biofilm development. However, we showed that during *Bacillus subtilis* biofilm development, induction of the DDR shuts off biofilm matrix genes [32] indicating an inverse correlation between DDR and biofilm development. These varied relationships are intriguing as cells have to balance different survival strategies, induction of DDR and biofilm formation, which could have downstream effects in treatment and clinical practices.

Here, we find that the relative change in the intracellular concentration of RecA upon DNA damage in *A. baumannii* is lower than that of *Escherichia coli*. We show that RecA influences biofilm

formation, and that a loss of RecA protein results in increased surface adherence and biofilm matrix formation with a higher sugar content. We also find that Δ*recA* cells within a biofilm are more resilient to antimicrobial action compared to the parental strain. The relationship between the DDR and biofilm in *A. baumannii* presents a novel challenge for controlling resistant infections.

Results

The relative induced intracellular concentration of RecA in *A. baumannii* ATCC17978 is lower to that of *E. coli*.

To determine the relative RecA intracellular concentrations in *A. baumannii* and *E. coli*, we purified *A. baumannii* RecA and *E. coli* RecA protein. We then performed an immunoblot of standard curves of the purified RecA from *E. coli* and from *A. baumannii* (Fig. S1A(i), B(i)). For this experiment, the degree to which the anti-RecA antibody recognizes *A. baumannii* RecA was determined first, since the antibody was raised against the *E. coli* RecA protein. We find that there is no significant difference in antibody recognition for the two proteins, which is supported by percent primary sequence identity between RecA from *A. baumannii* and *E. coli* (71%) (Fig. S1A(ii), B(ii)). Therefore, there was no need to account for differences in antibody recognition for RecA between the two bacteria.

It has been shown that in *E. coli*, RecA intracellular concentrations at basal level is in the ~1μM range and increases ~10-20 fold under DNA damage inducing conditions [33,34]. We find that under inducing conditions RecA concentrations in *E. coli* (Table 2) increase 20-fold, consistent with previous findings[33,34]. The assays that we performed showed that the basal intracellular RecA concentration in *E. coli* and *A. baumannii* are similar (see Table 2). However, upon DNA damage, the concentration of *A. baumannii* RecA is only 3-fold higher than basal levels, lower than the induced levels in *E. coli* [34] (Fig. 1). The lower induction we measured in RecA *A. baumannii* is in

agreement with the measurements of mRNA transcripts under DNA damaging conditions previously reported [35].

RecA concentration measurements in *A. baumannii* were performed in bulk cultures to directly compare with previous transcript measurements[35] and for ease of manipulation. It could be argued that the obtained measurements in induced cells is an underestimation. In *A. baumannii* there are two cell types formed in response to DNA damage, one with low and the other with high expression of *recA* [36]. Thus, bulk cultures would have a "dilution" factor that would correspond to the low expressing *recA* cells. Our estimation is that this factor would be at the most 20% of the total, which corresponds to the fraction of low *recA* cells when treated with 10X MIC of Cip [36]. Therefore, we conclude that under DNA damage inducing conditions, *A. baumannii* RecA intracellular concentrations are lower than in *E. coli*, and this may be related to unique regulatory elements that we found in *A. baumannii recA*[10].

RecA negatively influences biofilm formation in *A. baumannii* ATCC17978

A. baumannii ATCC 17978 Δ*recA* cells (*recA*::km, Table 1, [37]) grown in standard LB growth medium and shaking conditions consistently displayed observable growth on the sides of the glass tubes (Fig. 2A). In comparison, the parental ATCC 17978 wild-type strain (WT, Table 1) had little to no growth on the sides of the glass tubes (Fig. 2A). This observation suggested that *A. baumannii* Δ*recA* cells might produce more biofilm than WT cells. The Δ*recA* strain was previously characterized and shown to have no growth defects compared to the parental WT strain [37], though the biofilm phenotype was not investigated.

Pellicle biofilms, formed at the air liquid interface, were prepared with WT and Δ*recA* strains to visualize any phenotypic characteristics. Δ*recA* cells (Fig. 2B, middle panels) form a visible pellicle

at 24 hrs while WT *A. baumannii* cells form a visible pellicle (Fig. 2B, left panels) at 48 hrs. The WT pellicle at 48 hrs is smooth with striations, while the Δ*recA* pellicle is thicker and granular on the surface (Fig. S2). Cell adherence to the wells was measured by staining surface-attached cells with Crystal Violet as indicated in the methods section. We found that there is an increase in cell surface adherence in Δ*recA* biofilms both at 24 and 48 hrs compared to WT cells (Fig. 2C). At 72 hrs pellicles started to decline, which could be when biofilm disassembly begins (Fig. 2C). To confirm that the observed phenotypes were due to loss of RecA, the Δ*recA* strain was complemented *in trans* with an extrachromosomal copy of *recA* on a low copy plasmid (P_{recA}-*recA*) [10] (Fig 2B, right panels). At 24-72 hrs there is less observable pellicle formation in the complemented Δ*recA* strain, and the surface architecture is similar to WT (Fig. S2). Quantification of surface attachment confirms partial complementation (Fig. 2C). It is possible that gene dosage may somehow affect the surface attachment phenotype [38,39].

In addition to pellicles, biofilms are often studied as colonies (growth at the air-surface interface) [40]. Typically, robust colony biofilms have more wrinkling and surface architecture due to differential cell death and mechanical forces as a result of increased extracellular matrix [41]. At 48 hrs the Δ*recA* colony biofilm grown on solid LB medium had a rigid edge and surface wrinkles compared to the smooth WT colony biofilm (Fig. 3). The Δ*recA* strain colony biofilm complemented with a low copy number plasmid with the *recA* gene (Fig. 3), shows a mostly smooth colony.

These results suggest that RecA negatively influences biofilm development. To further test this relationship, we introduced a plasmid borne copy of *recA* (P_{recA}-*recA*, Table 1) into the WT strain (hereon referred to as $recA^{++}$). To make sure that the observed difference in biofilm attachment is due to RecA, we measured the intracellular concentration of RecA protein made by the $recA^{++}$ strain. We determined that the relative level of RecA in $recA^{++}$ is approximately 5-fold higher than both basal an and induced levels than the parental $recA^{+}$ strain (Fig. 4C, Table 2). We predicted the $recA^{++}$

biofilms would have fewer surface-attached cells than biofilms from WT or Δ*recA* cells. Indeed, Crystal Violet staining showed that the *recA*$^{++}$ strain at 48 h had significantly less surface-attached cells compared to the WT and Δ*recA* (Fig 4A&B).

Overall, the evidence supports an inverse correlation between RecA and biofilm formation. Surprisingly, it appears that small differences in intracellular RecA concentrations have large phenotypic effects. In *Escherichia coli* RecA intracellular concentrations are much higher, but RecA forms storage structures[42] which may limit the phenotypic effects of higher RecA. Given that the induced levels of RecA in the parental strain closely match that of the *recA*$^{++}$ strain (Table 2), our findings suggest that upon DDR induction, *A. baumannii* have decreased ability to form biofilms due to the relative change in RecA levels, especially in the high RecA cell type. It is possible that there may be *recA*-dependent genes being upregulated or downregulated that may affect biofilm development however this is yet to be determined. Due to the absence of a LexA homologue in *A. baumannii*, it is possible that RecA may serve different regulatory role in cells as has been previously suggested [35].

Δ*recA* cells form biofilms with both increased matrix and a shift toward higher total carbohydrate content

One possible explanation for the pellicle and colony biofilm phenotypes observed is that Δ*recA* cells produce more matrix or produce matrix earlier compared to WT cells. Pellicle biofilms from the Δ*recA* and WT strains at 24 hrs and 48 hrs were imaged by scanning electron microscopy (SEM) to observe if there was more matrix. To gain insights into the composition of the matrix, the SEM procedure included 2 different fixation methods (detailed in Materials and Methods). The first fixative (Fix 1) uses cationic dyes, which bind to negatively charged polysaccharides that are thus preserved for imaging. The second fixative (Fix 2) excludes cationic dyes, such that

exopolysaccharides are not preserved in the imaging [43]. There was noticeable difference between the matrix produced and biofilm structures of the WT and Δ*recA* strains (Fig. 5, Fix 1). At 24 hrs, Δ*recA* cells are embedded in an observable thick matrix, while the WT cells were not (Fig. 5, Fix 1). The biofilms formed by either strain appeared naked in the absence of polysaccharide preservation, which suggests that the matrix observed in Fix 1 is largely composed of polysaccharides (Fig. S3, Fix 2). At 48 hrs, the Δ*recA* cells are embedded in an even thicker matrix. The WT cells at 48 hrs (when these cells formed a visible pellicle; Fig. 2B) are more tightly packed than at 24 hrs with observable matrix. However, the WT cells do not appear to be as embedded within the matrix as the Δ*recA*, and they are preserved by Fix 2 at 48 hrs (Fig. S3). This finding suggested that Δ*recA* cells produce extracellular matrix earlier in their lifecycle that may have a different composition to matrix produced by WT cells.

To determine and quantify whether the differences in biofilm matrix between the WT and Δ*recA* strains were due to changes in polysaccharides, total extracellular matrix was extracted from stationary phase cells and carbohydrate content was measured. Δ*recA* cells had a nearly 3-fold increase in total carbohydrates compared the WT cells (Fig. S4). The complemented strain, Δ*recA w/PrecA-recA*, had a similar carbohydrate level to WT cells (Fig. S4). Most of the extracellular matrix or connections between the Δ*recA* cells (Fig. 5) are not visible with Fix 2 (Fig. S3, Fix 2), which suggests they are due to increased sugar content in the matrix. However, it is possible that in addition to increased sugar matrix content we may also be visualizing other phenomena, such as increased pili or nanotubes. Further investigation is needed to determine if this is the case.

Δ*recA* surface-attached cells withstand antibiotic treatment better than WT cells

Surface-attached WT and Δ*recA* biofilms were treated with the bactericidal aminoglycoside Gentamycin to determine if there were any differences in susceptibility compared to free-living cells. The choice of gentamycin was twofold: it is a clinically relevant bactericidal antibiotic for *Acinetobacter baumannii* infections that inhibits protein synthesis [44] and the MIC of exponentially growing cells is the same for both WT and Δ*recA* cells (1.88 μg/mL, error within 2-fold). Therefore, RecA does not impact gentamycin susceptibility in free-living cells. To measure the ability of Gentamycin to eradicate biofilms, the spent growth medium from 72 hr pellicle biofilms was removed and the remaining surface-attached cells were treated with different Gentamycin concentrations for 24 hrs in fresh growth medium (Fig. S5A). The next day cell density in the well dishes matched the MIC experiment of free-living exponential cells (Fig. S5B). The remaining surface-attached cells were stained with Crystal Violet. Strikingly, Crystal Violet staining showed that Δ*recA* cells withstood treatment better than WT cells (Fig. 6). For the Δ*recA* strain, the percentage of surface-attached cells remaining plateaus above 50% from 0.47–15 μg/mL of gentamycin (Fig. 6B). This represented an over 3-fold excess of Δ*recA* surface-attached cells remaining compared to the WT (at 15 μg/mL the percentage of WT surface-attached cells decreases to ~16%). To determine if the remaining surface-attached cells were viable, fresh medium was added after washing the antibiotic treated wells and incubated overnight at 37 °C. After incubation, all wells had visible growth (Fig. S5), indicating that remaining surface-attached cells are viable after gentamycin treatment. Growth, however, could have been the result of only a few living cells within the surface-attached population.

Therefore, to directly measure viability of surface-attached cells before and after gentamycin treatment, we performed LIVE/DEAD staining on remaining surface-attached cells and quantified the number of cells within 5 independent fields of view. We find that with no gentamycin treatment

and compared to the WT, the Δ*recA* strain had ~ 2-fold more viable surface-attached cells while in the *recA*$^{++}$ approximately half of them are found attached (Table 3). The gentamycin treated biofilms (Table 3) shows that though free living planktonic Δ*recA* is more sensitive than WT to antibiotic treatment[37], in a biofilm survives approximately 4-fold better than WT (Table 3). In the treated *recA*$^{++}$ strain, surface attached cells could not be recovered, which is consistent with its lowered ability to form biofilms (Table 3). Taking all of this data together, it seems that RecA influences biofilm development and its expression is inversely correlated with biofilm genes.

Discussion

Bacteria largely exist in the environment as biofilms[45]. Generally, cells in biofilms have different characteristics and gene expression profiles than their free-living planktonic counterparts. Here, we find that in *A. baumannii,* a key DNA damage response gene, *recA*, negatively influences biofilm development. Our results show that Δ*recA* cells have increased surface adherence while cells with excess RecA or induced levels of RecA (*recA*$^{++}$) have decreased adherence (Figs. 2-4, Table 3). We showed that RecA induced levels are lower than those of *E. coli* (Fig. 1, Table 2) and that differences in RecA intracellular concentration in the *recA*$^{++}$ cells have significant differences in phenotype (Fig 4). Moreover, the *recA*$^{++}$ cells, which mimic the induced concentrations of RecA (Fig 4), demonstrates the consequences of an active DDR on biofilm development.

The adherent cells produce more extracellular matrix earlier with greater total carbohydrate content (Fig. 5, Fig S4). Importantly, Δ*recA* biofilms on an abiotic surface are more resilient to antibiotic challenge (Fig. 6, Table 3). Moreover, we find that the antibiotic-challenged surface-attached cells are viable after antibiotic treatment (Table 3) providing additional evidence to the concept that biofilms protect cells from antibiotic exposure[14,15]. Thus, recurring infections may be the product of cells remaining adhered to equipment surfaces or implanted devices.

Aranda *et al.* phenotypically characterized the *ΔrecA* insertional mutant used in this study. Free-living *ΔrecA* cells had decreased survival during heat shock, desiccation, UV, and antibiotic treatment [37]. Importantly, the *ΔrecA* strain had much lower pathogenicity in a mouse model, indicating its importance in this process [37]. These results demonstrate the significance of RecA in survival and virulence in free-living planktonic cells. However, additional evidence [46] is consistent with our findings suggesting the importance of investigating both biofilm and free-living states. For example, it has been shown that strong *A. baumannii* biofilm formers are less frequently antibiotic resistant [46], consistent with low mutagenesis (i.e. low DDR or *recA* gene expression, and similar to Δ*recA*). However, these cells were more resistant to eradication [46], again consistent with the observation that Δ*recA* surface-attached cells are more difficult to eradicate with antibiotics. For example, certain antibiotics have lowered penetration of biofilms [14,15] and there is a nutrient gradient within the biofilm which leads to different metabolic states [47]. Thus, it is important to understand differences in gene expression, survival and phenotypic differences in both planktonic and biofilm states.

Our findings have intriguing impacts on the lifestyle of *A. baumannii*, especially in the clinic. We have previously shown that in response to DNA damage there is formation of two phenotypic subpopulations or cell types, one with low and one with high *recA* and DDR gene expression[36]. Therefore, whenever *A. baumannii* senses stress such as antibiotic treatment, there would be cells within the same clonal population that would favor biofilm formation (low *recA* expression) and cells that would favor mutagenesis (high *recA* expression) and biofilm dispersal. However, the molecular underlying mechanisms leading RecA to influence biofilm development remain to be identified.

These results demonstrate the complexity of treating pathogens such as *A. baumannii* with a DDR that does not follow the paradigm [10,36]. Recent work has highlighted the potential for clinical RecA inhibitors to potentiate the effect of antibiotics while hindering antibiotic resistance acquisition in

bacteria [48]. This indeed may be promising in the treatment of certain bacteria, as demonstrated in *E. coli* [48]. Our results show that for *A. baumannii*, removing mutagenic capacity may be an important avenue to explore to combat acquisition of resistant infections, but biofilm formation of strains in which RecA is inhibited may prove to be an unwanted side effect. It is thus important to understand the relationships between survival strategies in bacteria, and that different bacteria may have different responses to treatment, based on their fundamental biology.

Methods

Strains and Growth Conditions

Strains used are listed in Table 1. All bacterial cultures were routinely grown in LB medium, unless otherwise noted, and incubated at 37°C with shaking at 225 rpm for liquid cultures. YT medium is composed of 2.5g NaCl, 10g Tryptone and 1g Yeast extract per liter. The *A. baumannii w/* P_{recA}- *recA* strain was constructed by introducing the P_{recA}- *recA* plasmid into the WT strain, as described before[10].

Purification of His-tagged *A. baumannii* RecA

The *recA* gene from *A. baumannii* and *E. coli* were cloned into a pET-His6-TEV-LIC vector (plasmid 29653; Addgene, Cambridge, MA, USA) and introduced by transformation into DH5α *Escherichia coli* cells for plasmid maintenance. The induction of expression and purification methods are as those previously followed to purify RecA from *Escherichia coli* [33]. RecA purification was confirmed by SDS-PAGE.

Analysis of intracellular RecA concentration

Strains of *A. baumannii* and *E. coli* (Table 1) were used for these experiments. *A. baumannii* ATCC 17978 and an isogenic strain with a plasmid borne *recA* gene, pRecA[10], which we denote as $recA^{++}$,

was inoculated into LB broth with antibiotics as appropriate. Saturated cultures were diluted 1:50 (*recA*$^{++}$) or 1:100 (all others) and grown for 3 hours (hrs) to exponential phase. Ciprofloxacin (Cip) was used as before at 10x the MIC[10]. A parallel culture for each strain was left untreated. 2, 4, and 6 mL samples of cells were collected by centrifugation and lysed with BugBuster (Novagen) and Pierce Universal Nuclease according to manufacturer's instructions. Cell free lysates of treated and untreated samples were quantified following manufacturer's instructions (BioRad) and were combined with 5X Laemmli buffer to the same total volume and denatured at 75ºC for 10 minutes. The samples were separated on a 12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane [49]. The blot was developed as before [49] using an anti-RecA antibody (Abcam) at a 1:10,000 dilution [11] and a goat anti-rabbit antibody (Abcam) diluted 1:40,000. A ChemiDoc MP imaging system (BioRad) was used to detect the signal from the blots. Three biological replicates were used to estimate the relative µg of RecA per µg of total protein used.

The density of the RecA bands was quantified by ImageJ [11]. The RecA signal from Cip treated and untreated samples were compared to a standard of purified RecA protein from either *A. baumannii* or *E. coli*. The numbers obtained from the ImageJ quantification were divided by the total concentration of protein loaded in each lane.

Pellicle & Colony Biofilm Formation

To form pellicle biofilms, that is biofilms formed at the air-liquid interface, cells from an exponential culture were inoculated at a 1:1000 dilution into YT liquid medium in either 12 or 24-well non-tissue culture treated (do not contain a coating to change surface properties) polysterene plates. The same number of cells, adjusted based on optical density, were added to each well. Plates were then incubated statically at 25°C. A standard Crystal violet staining procedure was used to quantify adherence to the polystyrene surface [50]. For colony biofilms, 5 µL of a saturated

bacterial culture were spotted on to a solid LB medium plate and incubated for 48 hrs at 37°C. Images of biofilms were taken with a Leica MSV269 dissecting scope and a Leica DMC2900 camera, using the same settings.

Coverslip Biofilms and SEM microscopy

Biofilms for SEM were prepared by collecting pellicle biofilms on a glass coverslip treated with a solution of 0.1 mg/mL of polylysine (Fisher Scientific). The coverslips with the biofilms were deposited in wells with either fixative 1 or 2 for at least 24 hrs. Fixative 1 is composed of 25% Glutaraldehyde, 0.1 M Na-Cacodylate buffer (pH 7.2), 0.15% Alcian Blue and 0.15% Safranin. Fixative 2 is composed of 25% Glutaraldehyde and 0.1 M Na-Cacodylate buffer (pH 7.2). Additional treatment of the coverslips and observation of the biofilms was performed at the Northeastern University Electron Microscopy Core Facility.

Sugar Extraction Assay

Total extracellular matrix was extracted and total carbohydrate content was quantified using a modified protocol from Jiao et al. 2010 [51]. Briefly, 500 µL of YT medium saturated cultures of the *A. baumannii* strains were centrifuged at 13,000 rpm for 1 minute in pre-weighed micro centrifuge tubes and washed twice with 1X PBS; supernatant was discarded. Cell pellets were dried at 95°C and reweighed. This process was repeated until all dried pellets were approximately equal in weight (differences no more than 20% from each other). Pellets were reconstituted in 100 µL of 1X PBS and 400 µL of a 0.1 M sulfuric acid solution was added to each tube. Cell suspensions were mixed in a vortex every 10 minutes for 1 hr. Then, 250 µL of concentrated sulfuric acid (95% minimum) was added, followed by 50 µL of freshly made 10% phenol solution in water. This mixture was incubated at 95°C for 5 minutes. The absorbance at 492 nm of each sample was determined in a Biotek plate reader (Synergy HT). This measurement represents total carbohydrates. The absorbance per gram of

dry cell was normalized to the values obtained with the parental strain for each of 3 biological replicates. An unpaired two-tailed T-Test was used for statistical analysis relative to the parental strain; * = P<0.05

MIC and Biofilm Eradication Assays, and Live/Dead determination.

Standard assays were used to test for MIC[52]. Pellicles were set up as described above and incubated statically at 25°C for the number of hours indicated in the respective figure legends, at which time cells that were not stuck to the wells' surface were removed, and wells washed three times with 1X PBS. YT medium containing Gentamycin, at a range of concentrations shown in the respective figures was added to each washed well. The plate was then incubated statically for 24 hrs at 25°C, after which the spent medium was removed, and wells were washed three times with PBS. Crystal Violet staining was then carried out as before[50]. LIVE/DEAD staining was performed on resuspended surface-attached cells, and microscopy performed as before[53]. The stained cells from surface- attached cells of the WT, Δ*recA*, and *recA*$^{++}$ after gentamycin treatment were mechanically disrupted to dislodge attached cells. This methodology did not affect cells' integrity. The number of total cells obtained from the surface-attached fraction varied from strain to strain due to their difference in ability to attach to surfaces. The total number of cells and their viability was determined by counting 5 independent microscope fields from each of the strains.

Author Contributions

V.G.G., and C.C. initiated research. B. I. performed preliminary experiments. C.C., P.M., M.D., W.F. and V.G.G. performed experiments and analyzed data. C.C. and V.G.G. wrote the manuscript.

Competing Interests

The authors declare no competing interests.

Funding

Margaret Downs was funded by the Undergraduate Research and Fellowships office at Northeastern University.

Acknowledgments

We would like to acknowledge members of the Godoy and Chai labs at Northeastern University for helpful comments and discussion. We would like to thank G. Bou from Servicio de Microbiologia, Complejo Hospitalario Universitario, La Coruña, Spain for the Δ*recA* strain.

Figure Legends

Figure 1. *A. baumannii* RecA DNA-damage induced intracellular concentration is lower than that of *E. coli*.

Immunoblot comparing RecA under basal and DNA-damage conditions in *E. coli* and *A. baumanii*. *A. baumannii* reaches lower induced RecA levels than *E. coli*. Representative blots are shown of experiments preformed at least three times. (+) indicates treatment: exponential phase cells grown in LB for 3 hours and treated for three additional hours with 10X MIC of Ciprofloxacin (cip). (-) indicates no treatment: cells are grown at the same time and under the same conditions as (+) but with no cip. Quantitative data is provided in Table 2.

Figure 2. RecA negatively influences surface attachment and pellicle biofilm development

(A) Saturated Δ*recA* cell cultures show growth on the side surface of glass test tubes compared to WT cells. (**B)** Δ*recA* forms visible pellicles biofilms at the air-liquid interface earlier than its isogenic WT counterpart. Biofilm pellicles formed by the WT, Δ*recA*, and Δ*recA* with a plasmid

borne copy of *recA* driven by its own promoter, P_{recA}-*recA*, strains were prepared as indicated in the Materials and Methods section and imaged at 24, 48 and 72 hrs. Representative images are shown. (**C)** Δ*recA* cells adhere to the abiotic surface better than WT. Absorbance at 570 nm for Crystal Violet staining of polystyrene surface-adhered cells at 24, 48 and 72 hrs in YT medium. Experiments were performed in triplicate and error bars represent standard deviation.

Figure 3. RecA influences colony biofilm architecture

Colony biofilms for WT, Δ*recA* and the complemented Δ*recA w/* P_{recA}-*recA* strains on solid LB medium and imaged at 48 hrs.

Figure 4. Higher intracellular RecA present in the RecA^{++} strain result in significantly lower biofilm formation than Δ*recA* and WT.

(**A**) Absorbance at 570 nm for Crystal Violet staining of polystyrene surface-adhered cells at 48 hrs show that RecA is inversely correlated with biofilm formation. **(B)** Inset showing data for WT and RecA^{++} strains. Error bars represent the standard deviation of the mean of 4 replicate wells. * represents significance $p<0.05$ in a non-parametric T test, and ** represents a significance of <0.005. (**C**) A plasmid borne copy of RecA in WT *A. baumannii* results in approximately 3 to 5-fold higher RecA intracellular concentration. Representative images of immunoblots performed with whole cell extracts of the WT (RecA) and the strain with the plasmid borne *recA* (RecA^{++}). The *recA* gene in the plasmid is expressed under its own promoter [10].

Figure 5. Δ*recA* biofilms have increased observable matrix compared to WT biofilms

Scanning electron microscopy (SEM) of WT and Δ*recA* biofilms collected on coverslips at 24 and 48 hrs, using Fixative 1 (as described in Materials and Methods), which preserves polysaccharides. Scale bar represents 3μm.

Figure 6. Biofilm formation influences antibiotic challenge

(A) Images after Crystal Violet staining of 72 hr biofilms formed after 24 hrs of Gentamycin treatment at concentrations below and above the MIC measured for free-living planktonic cells. **(B)** Percentage eradication of biofilms treated with Gentamycin at a range of concentration for WT and Δ*recA* cells. Experiments were performed in triplicate. Error bars represent standard deviation.

Table 1

Strain	**Description**	**Reference**
A. baumannii	ATCC17978 Parental strain	American Type Culture Collection
A. baumannii Δ*recA*	ATCC17978 *recA::km*	Gift of G. Bou ([37])
A. baumannii Δ*recA w/* P_{recA}- *recA*	ATCC17978 *recA::km* w/ pNLAC1 containing *recA* (A1S_1962) promoter–*recA*, Tet^R	[10]
A. baumannii w/ P_{recA}- *recA*	ATCC17978 w/ pNLAC1 containing *recA* (A1S_1962) promoter–*recA*, Tet^R	This work
Escherichia coli MG1655	ATCC 700926	American Type Culture Collection

Table 2

Relative RecA concentration in *A. baumannii*

	Basal[a]	Induced[a]	Relative Fold induction
A. baumannii	$2x\ 10^{-5}$	$6x10^{-5}$	~3
A. baumannii recA^{++}	$6x10^{-5}$	$2.5x10^{-4}$	~4
E. coli	$2x10^{-5}$	$5x10^{-4}$	~20

[a]µg of RecA/µg of total protein

Table 3

Viability of surface attached cells

Strain/Treatment	**Total number of attached live cells***	**Ratio to WT**
WT/No Gm	1046	**1**
Δ*recA*/No Gm	2366	**2.3**
recA^{++}/No Gm	627	**0.6**
WT/Gm[a]	293	**1**
Δ*recA*/Gm[a]	1046	**3.6**
recA^{++}/Gm[a]	NF	**_**

[a]15µg/mL; NF, None found; * in 5 fields

References

1. Rice, L. B. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J. Infect. Dis.* **197**, 1079–81 (2008).

2. Peleg, A. Y., Seifert, H. & Paterson, D. L. Acinetobacter baumannii: Emergence of a successful pathogen. *Clin. Microbiol. Rev.* **21**, 538–582 (2008).

3. Wendt, C., Dietze, B. & Dietz, E. Survival of Acinetobacter baumannii on Dry Surfaces. *J. Clin. Microbiol.* **35**, 1394–1397 (1997).

4. Smith, M. G. *et al.* New insights into Acinetobacter baumannii pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev.* **21**, 601–14 (2007).

5. Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13**, 42–51 (2014).

6. Cirz, R. T. *et al.* Complete and SOS-mediated response of Staphylococcus aureus to the Antibiotic Ciprofloxacin. *J. Bacteriol.* **189**, 531–539 (2007).

7. Fuchs, R. P. & Fujii, S. Translesion DNA Synthesis and Mutagenesis in Prokaryotes. *Cold Spring Harb. Perspect. Biol.* 1–22 (2013).

8. Little, J. W. & Mount, D. W. The SOS Regulatory Escherichia coli System of Review. **29**, 1982 (1982).

9. Robinson, A. *et al.* Essential biological processes of an emerging pathogen: DNA replication, transcription, and cell division in Acinetobacter spp. *Microbiol. Mol. Biol. Rev.* **74**, 273–97 (2010).

10. Ching, C., Gozzi, K., Heinemann, B., Chai, Y. & Godoy, V. G. RNA-Mediated cis Regulation in Acinetobacter baumannii Modulates Stress-Induced Phenotypic Variation. *J. Bacteriol.* **199**, 1–15 (2017).

11. Norton, M. D., Spilkia, A. J. & Godoy, V. G. Antibiotic Resistance Acquired through a DNA Damage-Inducible Response in Acinetobacter baumannii. *J. Bacteriol.* **195**, 1335–45 (2013).

12. O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm Formation as Microbial Development. 49–79 (2000).

13. Espinal, P., Marti, S. & Vila, J. Effect of biofilm formation on the survival of Acinetobacter baumannii on dry surfaces. *J. Hosp. Infect.* **80**, 56–60 (2012).

14. Anderl, J. N. & Franklin, M. J. Role of Antibiotic Penetration Limitation in Klebsiella pneumoniae Biofilm Resistance to Ampicillin and Ciprofloxacin. *Antimicrob. Agents Chemother.* **44**, 1818–1824 (2000).

15. Singh, R., Sahore, S., Kaur, P., Rani, A. & Ray, P. Penetration barrier contributes to bacterial biofilm-associated resistance against only select antibiotics , and exhibits genus- , strain- and

antibiotic-specific differences. *Pathog. Dis.* 2–7 (2016). doi:10.1093/femspd/ftw056

16. Tomaras, A. P., Dorsey, C. W., Edelmann, R. E. & Actis, L. A. Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: Involvement of a novel chaperone-usher pili assembly system. *Microbiology* **149**, 3473–3484 (2003).

17. Brossard, K. A. & Campagnari, A. A. The Acinetobacter baumannii Biofilm-Associated Protein Plays a Role in Adherence to Human Epithelial Cells. *Infect. Immun.* 228–233 (2012). doi:10.1128/IAI.05913-11

18. Loehfelm, T. W., Luke, N. R. & Campagnari, A. A. Identification and characterization of an Acinetobacter baumannii biofilm-associated protein. *J. Bacteriol.* **190**, 1036–1044 (2008).

19. He, X. *et al*. Biofilm formation caused by clinical acinetobacter baumannii isolates is associated with overexpression of the AdeFGH efflux pump. *Antimicrob. Agents Chemother.* **59**, 4817–4825 (2015).

20. Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A. & Actis, L. A. Characterization of a two-component regulatory system from Acinetobacter baumannii that controls biofilm formation and cellular morphology. *Microbiology* **154**, 3398–3409 (2008).

21. Farrow, J. M., Wells, G. & Pesci, E. C. Desiccation tolerance in Acinetobacter baumannii is mediated by the two-component response regulator BfmR. *PLoS One* **13**, 1–25 (2018).

22. Russo, T. A. *et al*. The Response Regulator BfmR Is a Potential Drug Target for Acinetobacter baumannii . *mSphere* **1**, 1–19 (2016).

23. Choi, A. H., Slamti, L., Avci, F. Y. & Pier, G. B. The pgaABCD Locus of Acinetobacter baumannii Encodes the Production of Poly- β -1-6- N -Acetylglucosamine , Which Is Critical

for Biofilm Formation . *J. Bacteriol.* **191**, 5953–5963 (2009).

24. Hardouin, J., Chabane, Y. N., Marti, S. & Rihouey, C. Characterisation of Pellicles Formed by Acinetobacter baumannii at the Air-Liquid Interface. *PLoS One* **9**, (2014).

25. Bales, P. M., Renke, E. M., May, S. L., Shen, Y. & Nelson, D. C. Purification and Characterization of Biofilm-Associated EPS Exopolysaccharides from ESKAPE Organisms and Other Pathogens. *PLoS One* **8**, (2013).

26. Sahu, P. K., Iyer, P. S., Oak, A. M., Pardesi, K. R. & Chopade, B. A. Characterization of eDNA from the Clinical Strain Acinetobacter baumannii AIIMS 7 and Its Role in Biofilm Formation. *Sci. World J.* **2012**, (2012).

27. Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. Antibiotics as intermicrobial signaling agents. *Proc. Natl. Acad. Sci.* **103**, (2006).

28. Takajashi, A., Yomoda, S., Ushijima, I. & Inoue, M. Orfloxacin, norfloxacin and ceftazimide increase the production of alginate and promote the formation of biofilm of Pseudomonase aeruginosa in vitro. *J. Antimicrob. Chemother.* (1995).

29. Inagaki, S., Fujita, K., Nagayama, K., Funao, J. & Effects, O. T. Effects of recombinase A deficiency on biofilm formation by Streptococcus mutans. *Oral Microbiol. Immunol.* 104–108 (2009).

30. Costa, S. B. *et al.* Adherence to abiotic surface induces SOS response in Escherichia coli K-12 strains under aerobic and anaerobic conditions. *Microbiology* 1964–1973 (2014). doi:10.1099/mic.0.075317-0

31. Walter, B. M., Cartman, S. T., Minton, N. P., Butala, M. & Rupnik, M. The SOS Response

Master Regulator LexA Is Associated with Sporulation, Motility and Biofilm Formation in Clostridium difficile. *PLoS One* **10**, e0144763 (2015).

32. Gozzi, K. *et al.* Bacillus subtilis utilizes the DNA damage response to manage multicellular development. *npj Biofilms Microbiomes* **3**, 8 (2017).

33. Tashjian, T. F., Lin, I., Belt, V., Cafarelli, T. M. & Godoy, V. G. RNA primer extension hinders DNA synthesis by Escherichia coli mutagenic DNA polymerase IV. *Front. Microbiol.* **8**, 1–9 (2017).

34. Bell, J. C. & Kowalczykowski, S. C. Mechanics and Single-Molecule Interrogation of DNA Recombination. *Annu. Rev. Biochem.* **85**, 193–226 (2016).

35. Hare, J. M., Ferrell, J. C., Witkowski, T. a & Grice, A. N. Prophage induction and differential RecA and UmuDAb transcriptome regulation in the DNA damage responses of Acinetobacter baumannii and Acinetobacter baylyi. *PLoS One* **9**, e93861 (2014).

36. MacGuire, A. E. *et al.* Activation of phenotypic subpopulations in response to ciprofloxacin treatment in Acinetobacter baumannii. *Mol. Microbiol.* **92**, 138–52 (2014).

37. Aranda, J. *et al.* Acinetobacter baumannii RecA protein in repair of DNA damage, antimicrobial resistance, general stress response, and virulence. *J. Bacteriol.* **193**, 3740–7 (2011).

38. Kuhl, S. A., Zimmer, J. A. & Rohatgi, P. Complementation of bacteriophage induction and recombination defects in Escherichia coli RecA- mutants by expression of the cloned T4 bacteriophage uvsX gene. *Curr. Microbiol.* **46**, 88–93 (2003).

39. Korch, S. B. & Hill, T. M. Ectopic overexpression of wild-type and mutant hipA genes in

Escherichia coli: Effects on macromolecular synthesis and persister formation. *J. Bacteriol.* **188**, 3826–3836 (2006).

40. Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. & Kolter, R. Sticking together: Building a biofilm the Bacillus subtilis way. *Nat. Rev. Microbiol.* **11**, 157–168 (2013).

41. Asally, M. *et al.* Localized cell death focuses mechanical forces during 3D patterning in a biofilm. *Proc. Natl. Acad. Sci.* **109**, 18891–18896 (2012).

42. Ghodke, H. *et al.* Spatial and temporal organization of reca in the escherichia coli dna-damage response. *Elife* **8**, 1–37 (2019).

43. Erlandsen, S. L., Kristich, C. J., Dunny, G. M. & Wells, C. L. High-resolution Visualization of the Microbial Glycocalyx with Low-voltage Scanning Electron Microscopy□: Dependence on Cationic Dyes The Journal of Histochemistry & Cytochemistry. *J. Histochem. Cytochem.* **52**, 1427–1435 (2004).

44. Tangy, F., Moukkadem, M., Vindimian, E., Capmau, M.-L. & Le Goffic, F. Mechanism of action of gentamicin components: Characteristics of their binding to Escherichia coli ribosomes. *Eur. J. Biochem.* **386**, 381–386 (1985).

45. Costerton, J. W. *et al.* Microbial biofilms. *Annu. Rev. Microbiol.* 711–745 (1995).

46. Wang, Y., Huang, T., Yang, Y., Kuo, S. & Chen, C. Biofilm formation is not associated with worse outcome in Acinetobacter baumannii bacteraemic pneumonia. *Sci. Rep.* 1–10 (2018). doi:10.1038/s41598-018-25661-9

47. Werner, E. *et al.* Stratified Growth in Pseudomonas aeruginosa Biofilms. *Appl. Environ. Microbiol.* **70**, 6188–6196 (2004).

48. Alam, K. *et al*. RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance Article RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance. *Cell Chem. Biol.* **23**, 381–391 (2016).

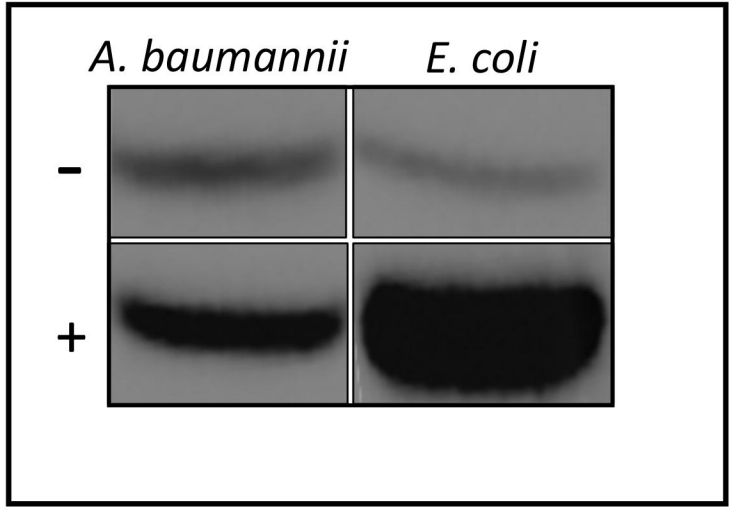
49. Cafarelli, T. M. *et al.* A single residue unique to dinb-like proteins limits formation of the polymerase IV multiprotein complex in Escherichia coli. *J. Bacteriol.* **195**, 1179–1193 (2013).

50. Chen, Y., Gozzi, K., Yan, F. & Chai, Y. Acetic acid acts as a volatile signal to stimulate bacterial biofilm formation. *MBio* **6**, 1–13 (2015).

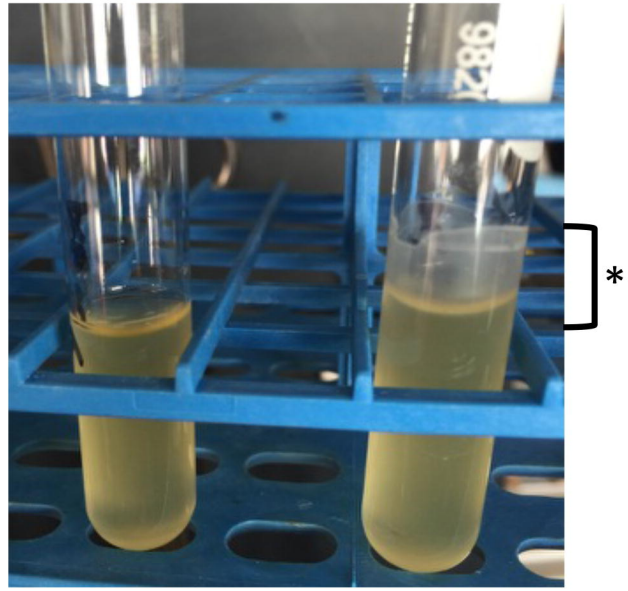
51. Jiao, Y. *et al.* Characterization of Extracellular Polymeric Substances from Acidophilic Microbial Biofilms □ †. *Appl. Environ. Microbiol.* **76**, 2916–2922 (2010).

52. Andrews, J. M. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* 5–16 (2001).

53. Ching, C. *et al.* Lon protease has multifaceted biological functions in *Acinetobacter baumannii*. *J. Bacteriol.* **201**, 1–12 (2018).

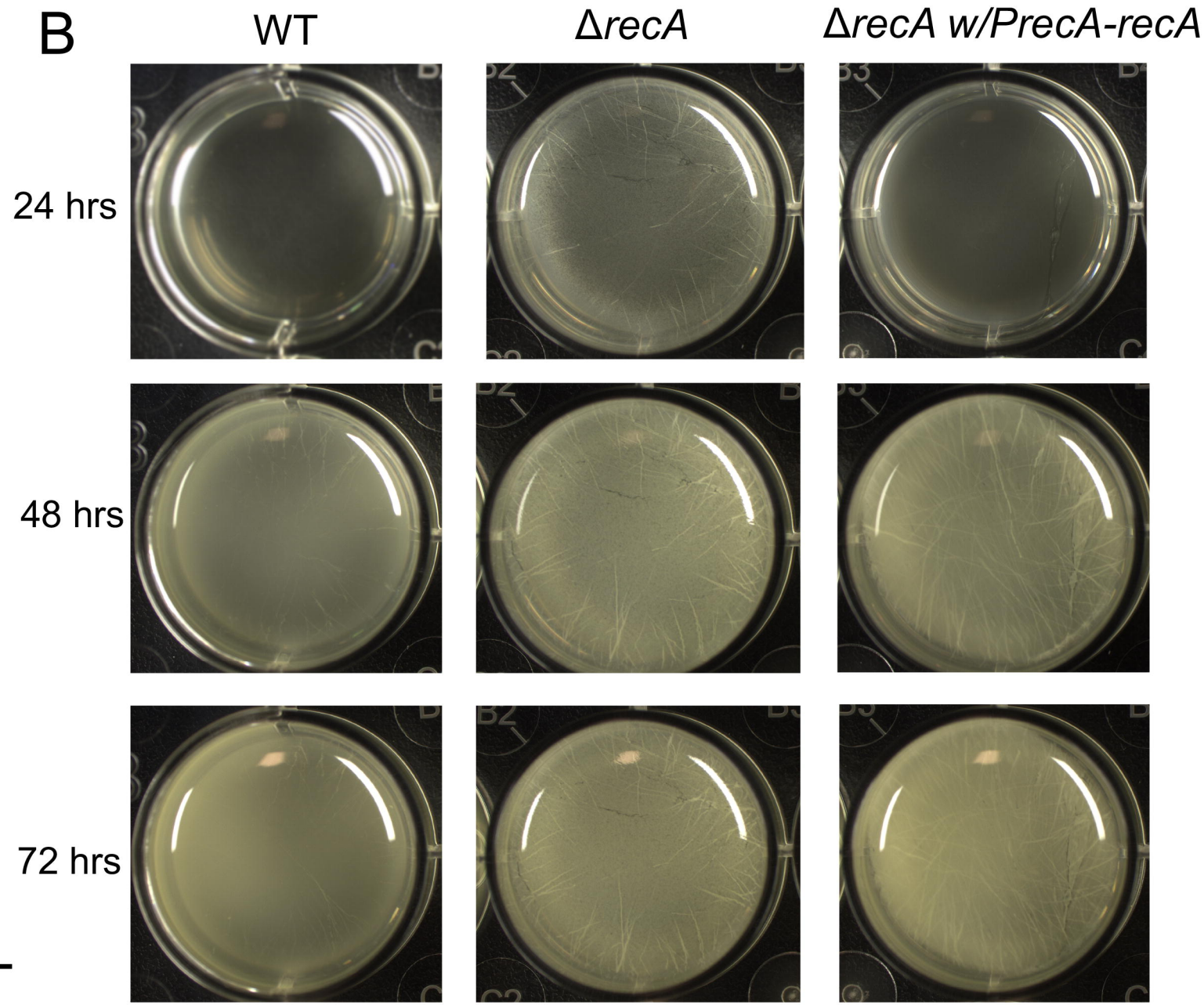

A. baumannii
E. coli
-
+

A

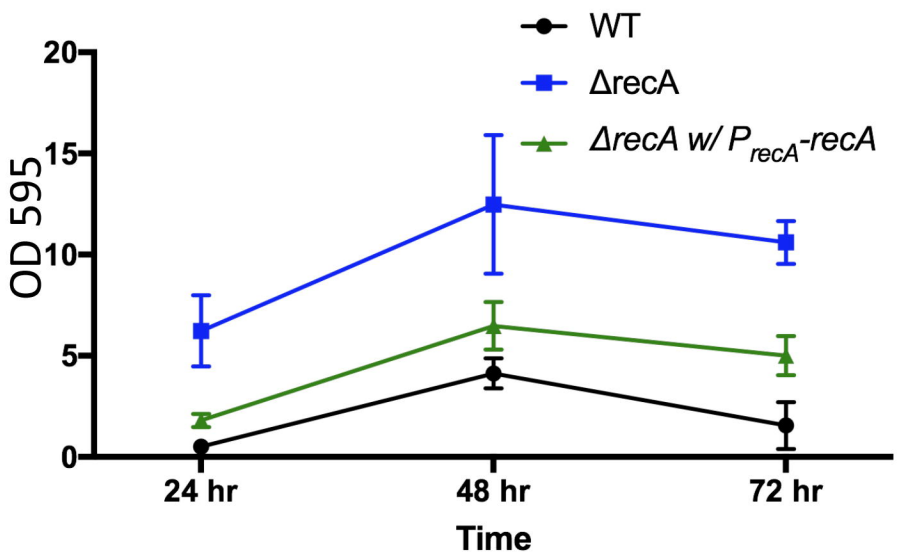

*

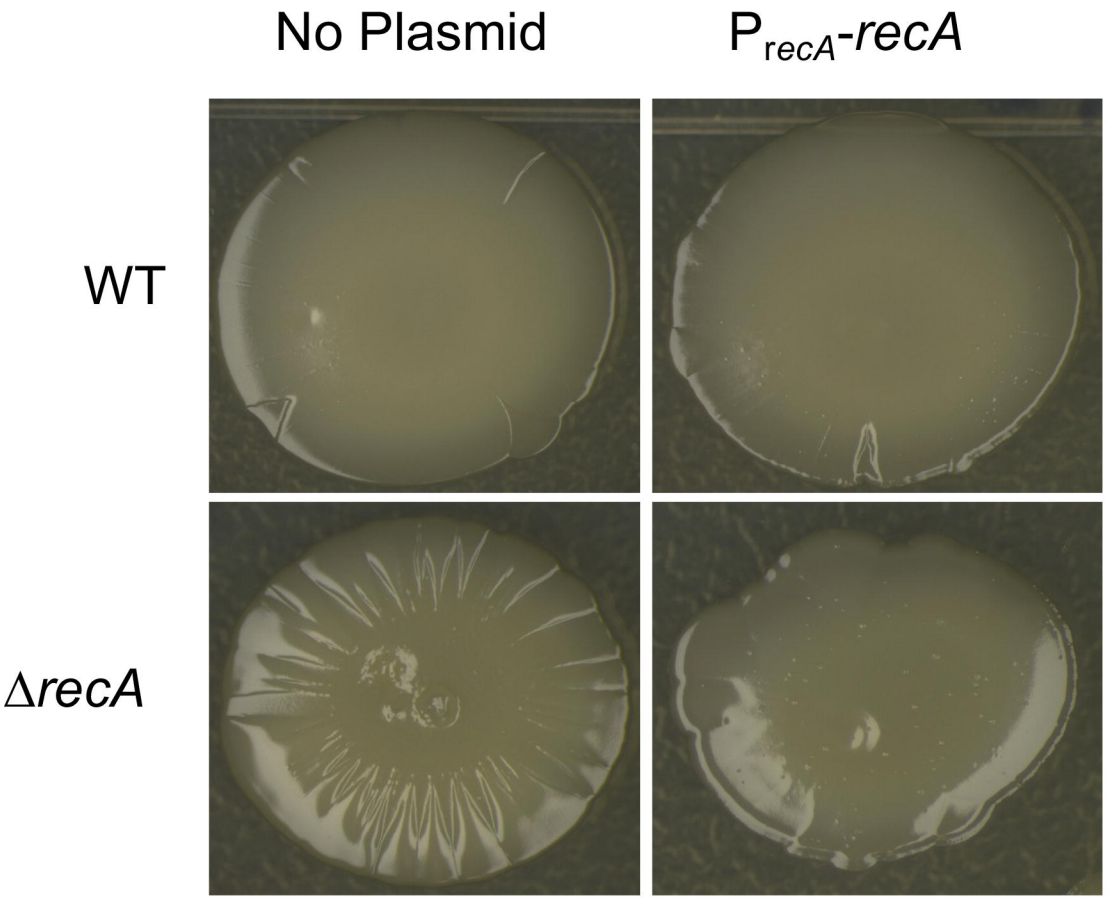
WT ΔrecA

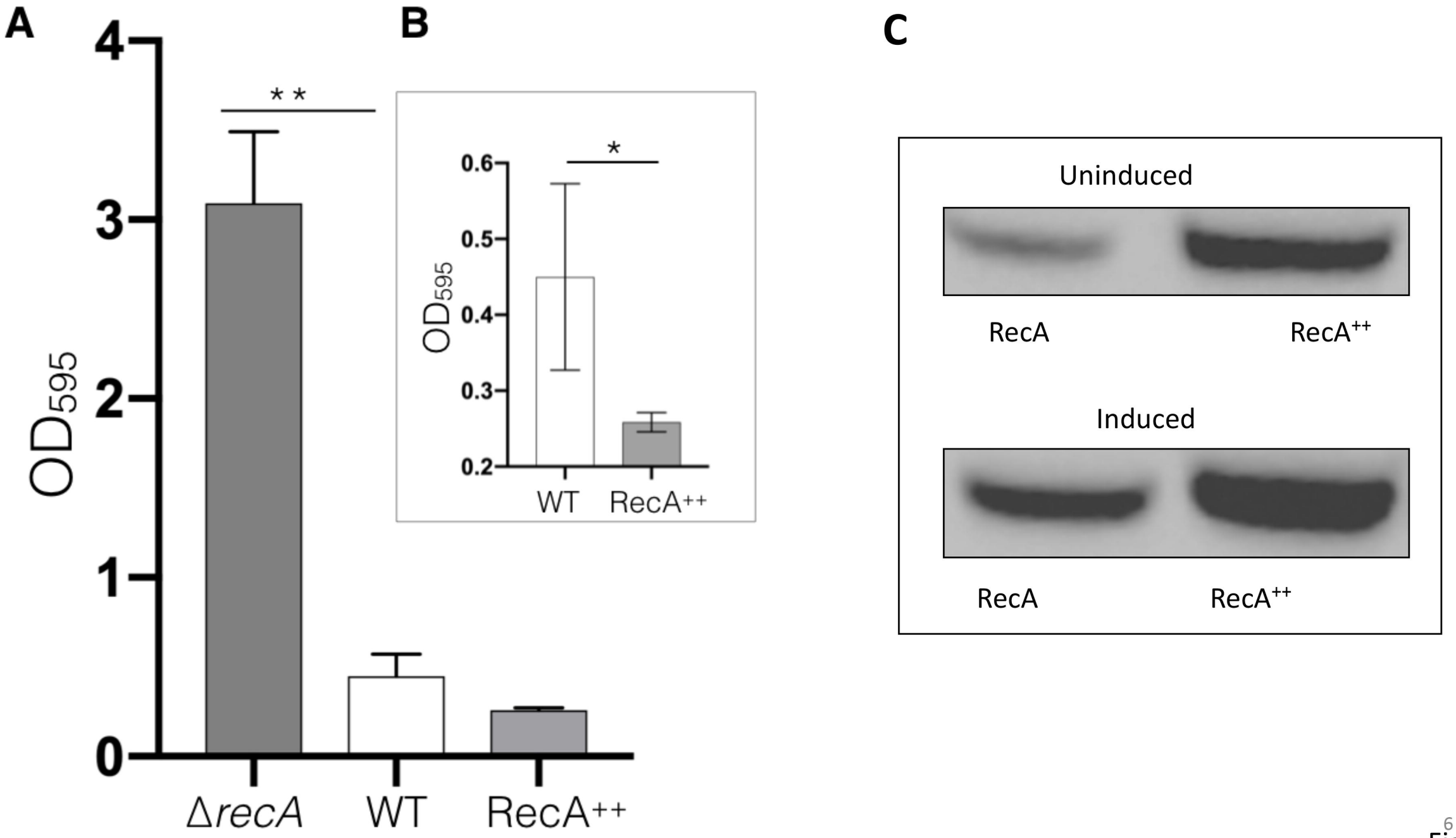
B

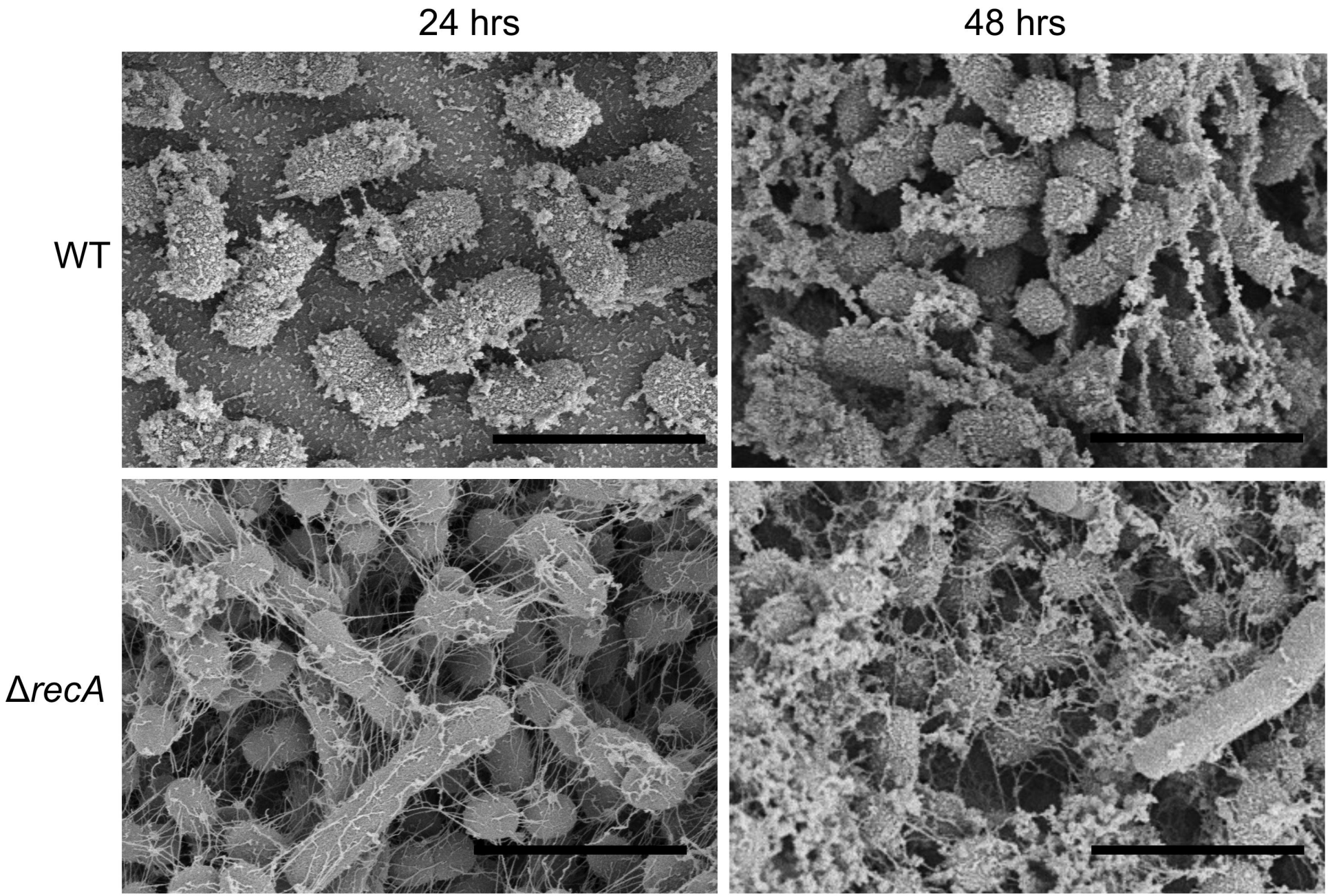

WT
ΔrecA
ΔrecA w/PrecA-recA
24 hrs
48 hrs
72 hrs

C

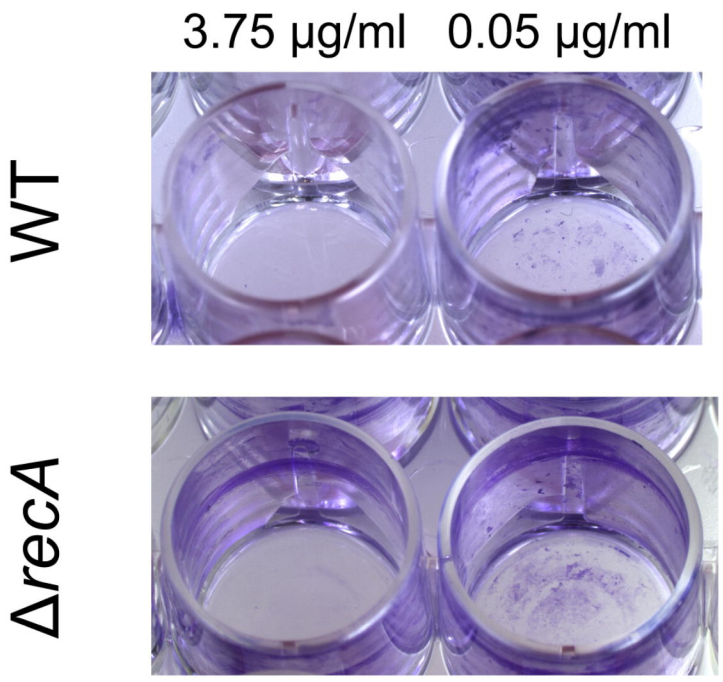

WT
ΔrecA
ΔrecA w/ PrecA-recA
OD 595
20
15
10
5
0
24 hr
48 hr
72 hr
Time


No Plasmid
P_{recA}-*recA*
WT
Δ*recA*

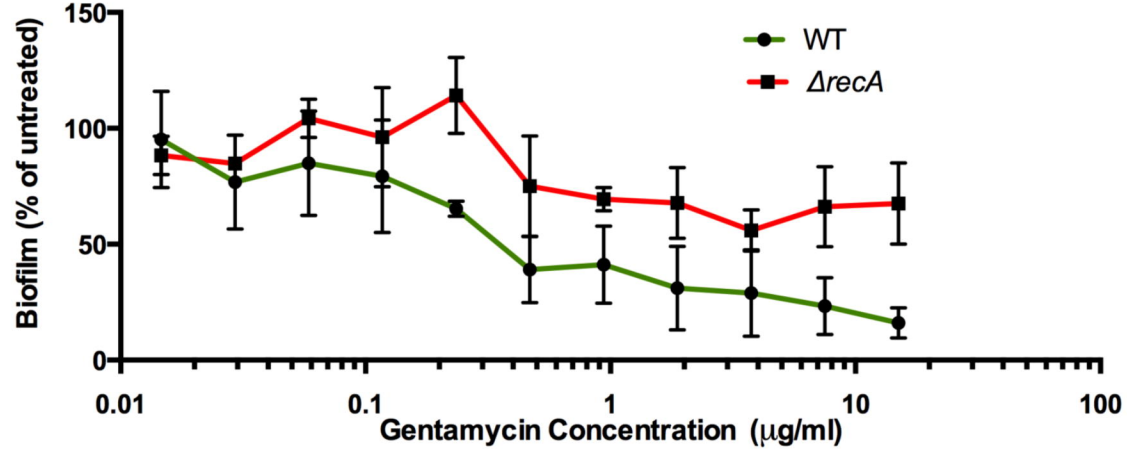

A
B
C
4
3
2
1
0
OD595
* *
ΔrecA
WT
RecA++
0.6
0.5
0.4
0.3
0.2
OD595
*
WT
RecA++
Uninduced
RecA
RecA++
Induced
RecA
RecA++


24 hrs
48 hrs
WT
ΔrecA

A


3.75 µg/ml
0.05 µg/ml
WT
ΔrecA

B


Biofilm (% of untreated)
150
100
50
0
0.01
0.1
1
10
100
Gentamycin Concentration (µg/ml)
WT
ΔrecA