The small DdrR protein directly interacts with the UmuDAb regulator of the mutagenic DNA damage response in *Acinetobacter baumannii*

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Running Header: DdrR interacts with the UmuDAb regulator

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Abstract

Acinetobacter baumannii poses a great threat in healthcare settings worldwide with clinical isolates displaying an ever-evolving multidrug-resistance. In strains of *A. baumannii*, expression of multiple error-prone polymerase genes is co-repressed by UmuDAb, a member of the LexA superfamily, and a small protein, DdrR. It is currently unknown how DdrR establishes this repression. Here, we use surface plasmon resonance spectrometry to show that DdrR forms a stable complex with the UmuDAb regulator. Our results indicate that the carboxy-terminal dimerization domain of UmuDAb forms the interaction interface with DdrR. Our *in vitro* data also show that RecA-mediated inactivation of UmuDAb is inhibited when this transcription factor is bound to its target DNA. In addition, we show that DdrR interacts with a putative prophage repressor, homologous to LexA superfamily proteins. These data suggest that DdrR modulates DNA damage response and prophage induction in *A. baumannii* by binding to LexA-like regulators.

Importance

We previously identified a 50-residue bacteriophage protein, gp7, which interacts with and modulates the function of the LexA transcription factor from *Bacillus thuringiensis*. Here we present data that indicates that the small DdrR protein from *A. baumannii* likely coordinates the SOS response and prophage processes by also interacting with LexA superfamily members. We suggest that similar small proteins that interact with LexA-like proteins to coordinate DNA repair and bacteriophage functions may be common to many bacteria that mount the SOS response.

Keywords

Induction of gene expression; Antibiotic resistance; Bacteriophage induction; DNA repair; SOS response

Introduction

The multidrug-resistant bacterium *Acinetobacter baumanii* has emerged as a global pathogen that thrives in nosocomial environments, and poses a growing threat to human health (1). There is therefore a need to understand the biochemical processes that enable rapid adaptation of *A. baumannii* to antibiotics, as these represent promising targets for intervention (2). Importantly, inhibiting the response to DNA damage might suppress the mechanisms that enable antibiotic resistance to arise in this pathogen (3, 4).

In *A. baumannii*, the induction of the DNA damage response depends on the RecA protein. However, the global transcription factor LexA, normally found in other bacteria, is absent from *Acinetobacter* spp., and instead, *A. baumannii* uses UmuDAb to regulate a part of its DNA damage response (5–7). In response to DNA damage, as shown in *E. coli* ectopically expressing UmuDAb, RecA forms an active nucleoprotein filament (RecA*), and this activates the self-cleaving activity of UmuDAb (8). The C-terminal dimerization domain (CTD) of UmuDAb is homologous to the UmuD component of the error-prone DNA polymerase V, with the catalytic core structurally similar to that of LexA superfamily members, carrying the Ser119 and Lys156 catalytic dyad, which catalyzes cleavage between residues Ala83 and Gly84, resulting in regulator autolysis (Fig. S1) (8). However, in contrast to other UmuD homologs, UmuDAb contains an N-terminal DNA-binding domain (8–10), which allows UmuDAb to bind to target promoters. In *A. baumannii* strain ATCC 17978, UmuDAb, negatively regulates its own expression, the divergently transcribed gene, *ddrR*, as well as six error-prone DNA polymerase V genes (5, 11). Induction of DNA polymerase V causes increased mutagenesis, which enables rapid adaptation to antibiotics that elicit the DNA damage response (6).

The *ddrR–umuDAb* locus is widespread among *A. baumannii* isolates (12), with *ddrR* encoding an ~9-kDa protein (DdrR) that is exclusive to *Acinetobacter* spp. DdrR has been identified as another regulator of the DNA damage response in *A. baumannii*, which can, according to transcriptomic analysis, repress or activate DNA-damage inducible gene expression (11). During normal bacterial growth, both DdrR and UmuDAb are required to tightly repress the expression of *ddrR*, *umuDAb* and the six error-prone *umuDC* DNA polymerase operons. Conversely, DdrR and UmuDAb co-activate the expression of certain prophage genes, such as the putative lytic cycle repressors (A1S_2037 and A1S_1144), of the cryptic phages CP9 and

CP5, respectively (11). A recent study using a two-hybrid assay failed to detect an UmuDAb-DdrR interaction (13), which may have been prevented by the protein domains fused to both UmuDAb and DdrR.

We previously reported the discovery of gp7, a ~7-kDa protein encoded by bacteriophage GIL01. Gp7 interacts with LexA to delay the DNA damage response, and this enables the GIL01 bacteriophage to establish a lysogenic state in its host, *B. thuringinesis*. Gp7 promotes these effects by enhancing LexA DNA binding and inhibiting its self-cleavage (14, 15). Although gp7 and DdrR do not share sequence similarity, we hypothesised that co-regulation by DdrR might occur through the formation of a complex with LexA-like regulators such as phage repressors and UmuDAb. Here, we demonstrate that DdrR does indeed interact with UmuDAb. Furthermore, we show that DdrR can interact with the catalytic CTD of UmuDAb and the putative repressor of cryptic prophage CP5, A1S_1144. This is also the first report showing that *A. baumannii* RecA can be activated by ssDNA (RecA*) and be used in *in vitro* studies. Our results suggest that DdrR functions in a mechanistically different manner to gp7, as DdrR did not enhance the DNA-binding or RecA*-mediated inactivation of UmuDAb.

Materials and methods

Protein expression and purification

The pET29b(+) expression vectors carrying the *A. baumannii* genes (American Type Culture Collection 17978; GenBank accession number, CP000521.1) used in this study, were prepared using DNA synthesis by Twist Bioscience. These constructs encode the full-length UmuDAb (gene ID: A1S_1389, InterPro classifies this sequence into the LexA/signal peptidase-like family with the InterPro accession number IPRO36286 and peptidase S24/S26A/S26B/S26C with the accession number IPRO15927), the UmuDAb CTD (gene ID: A1S_1389, genome coordinates 1631520 to 1631882, which contains residues from glycine [Gly84] of the putative cleavage site to the terminal arginine [Arg202]), UmuD_0636 (gene ID: A1S_0636), UmuD_1174 (gene ID: A1S_1174), A1S_1144 (gene ID: A1S_1144), A1S_2037 (gene ID: A1S_2037), RecA (gene ID: A1S_1962) and DdrR (gene ID: A1S_1388). All proteins were overexpressed as a fusion with an N-terminal thrombin-cleavable site and a hexahistidine tag.

Each protein was overexpressed in *Escherichia coli* strain BL21(DE3) (New England Biolabs). Cells were grown aerobically at 37°C in 500 mL lysogeny broth, supplemented with 50 µg mL^{-1} kanamycin, to an optical density at 600 nm of 0.6. Protein synthesis was induced by the addition of 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For cells expressing UmuDAb, UmuDAb CTD, UmuD proteins A1S_0636 and A1S_1174 or putative phage repressors (A1S_1144, A1S_2037), the cultures were induced for 4 h at 37°C. For cells expressing DdrR protein, cultures were cooled to 20°C before IPTG addition and were grown for an additional 18 h at 20°C before harvesting. Note that the synthesis of DdrR at 20°C increased the yield of the recombinant protein.

The proteins were affinity-purified using Ni-chelate chromatography (QIAGEN). Cell lysates were loaded onto pre-equilibrated columns, washed and proteins were eluted using buffers containing 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM (pre-equilibration), 20 mM (wash) and 250 mM (elution) imidazole, respectively. The eluted protein fractions were dialyzed, using dialysis membrane tubing (molecular weight cut-off, 3.5 kDa; Spectrum), to exchange the elution buffer directly against a buffer containing 10 mM Na_2HPO_4, pH 7.4, 300 mM NaCl, 1.8 mM KH_2PO_4, 2.7 mM KCl, 2 mM dithiothreitol, and protein samples were stored at -80°C. The resulting recombinant protein concentrations were determined using a spectrophotometer (NanoDrop1000; Thermo Scientific), with the extinction coefficients at 280 nm of 23950 M^{-1} cm^{-1} for UmuDAb, 16960 M^{-1} cm^{-1} for UmuDAb CTD, 15595 M^{-1} cm^{-1} for UmuD_0636, 18450 M^{-1} cm^{-1} for UmuD_1174, 31042 M^{-1} cm^{-1} for A1S_1144, 35410 M^{-1} cm^{-1} for A1S_2073, 15930 M^{-1} cm^{-1} for RecA and 11460 M^{-1} cm^{-1} for DdrR. Protein integrity and purity was analysed using SDS-PAGE (4-20% bis-Tris protein gels SurePAGE™ [GenScript] and stained with SimplyBlue SafeStain; Invitrogen). LexA proteins from *B. thuringiensis*, *Staphylococcus aureus* and *E. coli* were prepared as described previously (15).

Surface plasmon resonance

Surface plasmon resonance measurements were performed at the Infrastructural Centre for Analysis of Molecular Interactions in the Department of Biology, University of Ljubljana (Slovenia), on a Biacore T200 system (GE Healthcare) at 25°C. To investigate the interactions between proteins and the DNA fragment encompassing the wild-type or mutated *umuDAb* operator, a streptavidin sensor chip was used (SA; GE Healthcare). To prepare the different DNA

fragments, complementary primers (Sigma-Aldrich) (Table S1) were mixed at a 1:1.5 (long: short primer) mole-to-mole ratio in 50 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, and annealed using a temperature gradient decreasing from 94°C to 21°C. Approximately 40 resonance units (RU) of the annealed 28-37 bp DNA fragments that carried a 15-nucleotide overhang was hybridized to the complementary biotinylated S1 primer (Table S1) that was immobilized through the streptavidin–biotin interaction in flow cell 2 of the sensor chip. The DNA fragments were injected at a flow rate of 5 µL min^{-1}. The interactions of UmuDAb with the DNAs immobilized on the chip were measured by injecting the protein solutions in running buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.1 mg mL^{-1} bovine serum albumin, 0.005% surfactant P20) at 50 µL min^{-1} at the desired concentrations. Regeneration of the sensor surface was achieved by injection of 50 mM NaOH at 50 µL min^{-1} for 6 s. Data were analyzed using Biacore T200 Evaluation Software, and the equilibrium dissociation constant (K_D) of UmuDAb binding was determined by fitting the data to the steady-state affinity model in the dissociation phase at the time point 300 s after the end of sample injection.

To investigate the interaction of DdrR with UmuDAb, UmuDAb CTD, UmuD or A1S_1144, the proteins were immobilized on the carboxymethyl-dextran-coated gold surface of the CM5 sensor chip (GE Healthcare), used to assess a direct protein-protein interaction, as described previously (16). The final amounts of the immobilized ligands were 1600 RU for UmuDAb, 800 RU for UmuDAb CTD, 1600 RU for UmuD_0636, 1200 RU for UmuD_1174, 1200 RU for A1S_1144, 1000 RU A1S_2037 and 1200 RU for *B. thuringiensis* LexA, *S. aureus* LexA and *E. coli* LexA. DdrR was serially diluted in the same running buffer and used with the SA sensor chip. For titration reproducibility, each DdrR concentration tested (7.8-8000 nM) was injected across the immobilized proteins twice for 150 s at a low rate of 20 µL min^{-1}, followed by dissociation for 360 s. Regeneration of the sensor surface was achieved by injecting 0.07% sodium dodecyl sulfate for 8 s at a flow rate of 20 µL min^{-1}. The sensorgrams were doubly referenced for the flow cell 1 untreated surface response and the flow cell 2 buffer response. Data were analyzed using Biacore T200 Evaluation Software and K_Ds were determined by fitting the data in the dissociation phase (150 s after completion of sample injection) to the steady-state affinity model. This was possible due to the stable interaction of DdrR with the tested ligands and, therefore, allowed us to eliminate the contributions of possible unstable or nonspecific interactions in the association phase when determining affinity.

EMSA (electrophoretic mobility shift assay)

EMSA analysis, using purified UmuDAb and DdrR proteins, was carried out as detailed by Browning et al. (2006) (17). The 535 bp fragment of the *A. baumannii* genome carrying the UmuDAb and DdrR (*ddrR-umudAb*) promoter regions (genome coordinates 1630986-1631520) flanked by HindII and BamHI restriction sites, was synthesized by Twist Bioscience. The fragment was cloned into the high-copy plasmid pUC19 and propagated in *E. coli* DH5α. After HindII-BamHI enzymatic restriction, the fragment was separated from the plasmid by agarose gel electrophoresis and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). Purified *umuDAb* promoter fragment was end-labelled with P^{32} and approximately 0.5 ng of fragment was incubated with various amounts of each protein. The reaction buffer contained 20 mM HEPES (pH 8.0), 50 mM potassium glutamate, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mg ml^{-1} bovine serum albumin, 5% glycerol and 25 µg ml^{-1} herring sperm DNA. Samples were incubated in a final reaction volume of 10 µl for 20 min at 37°C and were immediately run on a 6% polyacrylamide gel (12 V cm^{-1}), containing 2% glycerol, in 0.25 x Tris-borate-EDTA buffer. Gels were analysed using a Bio-Rad PMI molecular imager with Quantity One software (Bio-Rad).

RecA*-dependent UmuDAb and putative phage repressors self-cleavage assay

Recombinant *A. baumannii* RecA (10 µM) was activated with the S2 primer (2.2 µM) (Table S1) in 10 mM Tris, pH 7.4, 2 mM $MgCl_2$, and 1 mM ATP-γ-S for 18 h on ice, as previously described (16). For non-activated RecA, S2 primer and ATP-γ-S were not added to the reaction mixture. UmuDAb (2 µM) was mixed with either specific or non-specific DNA in a ~1:4 molar ratio (monomer UmuDAb: target DNA site). The specific 37-bp DNA fragment encompassing its target site and the non-specific 29-bp DNA fragment with shortened target site were composed of complementary primers *ddrR-umuDAb* _L and *ddrR-umuDAb* _S or *ddrR-umuDAb* _m3_L and *ddrR-umuDAb* _m3_S, respectively (Table S1). To test whether DdrR has an effect on the self-cleavage of UmuDAb or putative phage repressors, 2 µM UmuDAb or one of the putative phage repressors (A1S_1144 or A1S_2037) were mixed with either DdrR in a molar ratio of ~1:5 (monomer of UmuDAb : monomer of DdrR) or an equal amount of DdrR storage buffer as a control. The cleavage reactions were performed in 10 mM Tris (pH 7.4), 5 mM $MgCl_2$, and 1 mM ATP-γ-S, at 37°C. Reaction time runs were initiated by adding 4 µL of

the activated RecA* (final concentration of 2 µM) per 16 µL of reaction mixture. At this point, the first 20 µL of sample was removed from the reaction mixture and designated as sample zero minutes. Samples were then taken 5, 10, 20, and 40 minutes after the addition of RecA*. Reactions were stopped immediately after sample collection by addition of sample buffer (NuPAGE LDS; Invitrogen). The samples were analyzed on 4-20% bis-Tris protein gels (SurePAGE™; GenScript), stained (SimplyBlue SafeStain; Invitrogen) and washed. UmuDAb protein band intensity was quantified using the manual band quantification tool of GeneTools analysis software.

Results

DdrR interacts with LexA proteins from Gram-positive bacteria

Previously we showed that the gp7 co-repressor, was promiscuous in its interaction partners, complexing with the cognate *B. thuringiensis* LexA as well as LexA from *S. aureus* (15). As DdrR co-regulates the expression of DNA damage response genes with UmuDAb, we examined whether DdrR could also interact with LexA homologs from *B. thuringiensis* and *S. aureus* or the Gram-negative model bacterium, *E. coli*. Surface plasmon resonance (SPR) analysis confirmed that DdrR can interact stably with LexA from *B. thuringiensis* and *S. aureus* (Fig. 1A and B), binding with an intermediate affinity (apparent dissociation constant of ~715 and 745 nM, respectively) (Fig. S2A and B). However, there was no detectable interaction between DdrR and *E. coli* LexA (Fig. 1C and Fig. S2C), indicating that only LexA homologues from these Gram-positive bacteria possess a DdrR interaction surface.

DdrR binds stably to UmuDAb

To determine whether DdrR directly interacts with UmuDAb, we injected a wide range of DdrR concentrations over UmuDAb immobilized to a CM5 SPR chip. In contrast to gp7, which dissociates rapidly from LexA (14), the association of DdrR with UmuDAb was more stable based on a slower DdrR dissociation from UmuDAb (Fig. 2A). We also expressed and purified the CTD of UmuDAb and analysed its interaction with DdrR. DdrR interacted with CTD of UmuDAb, with a similar affinity (K_D of 319 nM) to full-length UmuDAb (K_D of 241 nM) (Fig.

S3, Fig. 2A and B). This is consistent with our previous observation that gp7 is able to interact with both full-length LexA and the LexA CTD (15).

Since the CTD of UmuDAb is homologous to both UmuD proteins found in *A. baumannii* (A1S_0636 and A1S_1174) and to putative phage repressors (A1S_1144 and A1S_2037), we also tested the binding of DdrR to these proteins. While binding of DdrR to both UmuD proteins and the A1S_2037 protein was not significant, (Fig. S4), it bound strongly (K_D of 239 nM) to the putative phage repressor A1S_1144 from the CP5 prophage (Fig. 2C, Fig. S3C).

DdrR does not interact with the umuDAb–ddrR promoter region

To understand better the characteristics of the DdrR protein we investigated the binding of DdrR in comparison to UmuDAb to DNA using SPR and electrophoretic mobility shift assay (EMSA). SPR analysis showed that UmuDAb binds to a chip-immobilized 37-bp fragment containing its operator site (found in the *ddrR-umuDAb* promoter region) with an apparent equilibrium constant (K_D) of 11.6 ± 1.9 nM (Fig. 3A). A previous report showed that substitutions in this site abolish UmuDAb binding in EMSA assays (5). Since SPR is able to detect weak interactions with high sensitivity and track the interaction dynamics in real time, we re-examined the interaction of UmuDAb with DNAs modified at the most critical operator nucleotides (Fig. 3B). In contrast to the previous EMSA study (5), our SPR analysis showed that UmuDAb interacts with the modified operators, but the resulting complexes are less stable than when UmuDAb binds the wild-type operator (Fig. 3B). We speculate that in the EMSA analysis (5), such an unstable interaction leads to complete dissociation of UmuDAb from the modified operator during the course of the experiment. In addition, we used SPR to investigate the binding of DdrR to the UmuDAb operator region and found that DdrR does not interact with this DNA (Fig. 3A). To investigate whether DdrR might interact upstream or downstream of the UmuDAb target site, and because SPR analysis limits the DNA probe to a size of ~200 bp, we performed an EMSA using a 535-bp DNA fragment encompassing the *ddrR-umuDAb* promoter region (Fig. 3C). UmuDAb bound this fragment to form a single protein/DNA complex, but DdrR alone did not bind this DNA probe (Fig. 3C). In addition, SPR confirmed that UmuDAb, but not DdrR, stably binds operators found in the promoter regions of error-prone DNA polymerase V components (gene IDs: A1S_0636, A1S_1174, A1S_2008 and A1S_2015) (Fig. 3D, Fig. S5), similarly to the operator found in the *ddrR-umuDAb* promoter region (Fig. 3A).

The RecA protein of A. baumannii activates self-cleavage of the DNA-unbound form of UmuDAb

To determine whether *A. baumannii* RecA forms an active nucleoprotein filament, we prepared two separate RecA activation mixtures with or without ATP and a ssDNA fragment, which are both required for RecA activation (18). Only the addition of activated RecA* (RecA in the complex with ATP and ssDNA) resulted in UmuDAb self-cleavage (Fig. 4A). We previously showed that the *Clostridium difficile* and *E. coli* LexA regulators do not undergo RecA*-induced self-cleavage when in the operator-bound state (19, 20). Thus, we investigated whether this was also the case of UmuDAb. We tested the ability of *A. baumanii* RecA* to induce self-cleavage of UmuDAb bound to either the UmuDAb operator or in the presence of non-specific DNA. In presence of RecA*, cleavage of operator-bound UmuDAb was strongly inhibited over time, with 60 % of uncleaved UmuDAb remaining 40 min after the addition of RecA*, compared to 13 % of uncleaved UmuDAb in presence of non-specific DNA (Fig. 4B). Thus, we conclude that DNA-bound UmuDAb is protected from RecA*-induced cleavage.

Next, we investigated whether DdrR can inhibit RecA*-mediated cleavage of free UmuDAb, as observed in the gp7-LexA interaction (14) and so pre-incubated DdrR with UmuDAb (5:1 mol:mol), before adding RecA*. The results in Figure 4C show that DdrR did not appreciably affect the inactivation of UmuDAb by RecA*, as the percentage of intact UmuDAb was, 2% and 5%, 40 min after the addition of RecA in the presence and absence of DdrR, respectively.

As the putative phage repressors A1S_1144 and A1S_2037 carry the residues characteristic of the S24 peptidase domain, which enable transcription regulator autolysis (Fig. S1), we investigated whether incubation of each protein with RecA* resulted in cleavage. Our *in vitro* data in Fig. S6 indicate that *A. baumanii* RecA* does not stimulate self-cleavage in either protein and that A1S_1144 and A1S_2037 may become inactivated by another mechanism.

Discussion

In most bacteria, DNA-damage-inducible genes are controlled by the LexA regulator (21). As DdrR and UmuDAb co-regulate the DNA damage response in *A. baumannii*, we postulated

that DdrR might function analogously to the GIL01 phage co-repressor gp7, which interacts with the LexA regulator of *B. thuringiensis* to enhance its DNA binding (14, 15). Hence, we sought to determine whether DdrR can interact with LexA regulators from relevant human and insect pathogens. SPR analysis confirmed that DdrR interacts with LexA from *B. thuringiensis* and *S. aureus* (Fig. 1A and B), but not with *E. coli* LexA (Fig. 1C). Thus, our results indicate that LexA regulators from Gram-positive Firmicutes possess a DdrR-like interaction interface that is absent from *E. coli* LexA. In view of the fact that UmuDAb is a member of the LexA superfamily, we hypothesized that DdrR might exert its co-regulatory functions through direct interaction with UmuDAb. We show that *A. baumannii* DdrR interacts directly with UmuDAb and its CTD (Fig. 2A and B) and, like gp7, is not a DNA-binding protein (Fig. 3A, C and D). In contrast to the gp7-LexA interaction (14), we found that DdrR dissociates less rapidly from UmuDAb (Fig. 2A). Furthermore, unlike gp7 and LexA in *B. thuringiensis* (14), DdrR did not interfere with RecA*-induced self-cleavage of UmuDAb (Fig. 4C). This suggests that DdrR exerts its co-regulatory function in a mechanistically different manner to gp7. Gp7 and DdrR are not homologous and yet both proteins bind to and regulate the response of LexA like regulators, suggesting that convergent evolution has taken place. Thus, it is possible that small proteins like DdrR and gp7 may exist in other bacteria to co-regulate and fine tune the SOS response.

Our in vitro experiments also show that DdrR interacts with the putative phage repressor A1S_1144 of phage CP5 (Fig. 2C), but not with the putative phage repressor A1S_2037 of phage CP9 (Fig. S4C). This suggests that the regulatory mechanisms of A1S_1144 and A1S_2037 proteins differ and that DdrR possibly affects phage CP5 gene expression (11) through interaction with A1S_1144. Although both putative repressors appear to carry the catalytic Ser-Lys dyad and the cleavage site residues that are conserved in LexA family proteins (Fig. S1) (22), we did not observe RecA*-induced autolysis of either protein (Fig. S6). This suggests that they may be inactivated when bound to the target DNA, as observed for the cI repressor of the temperate bacteriophage 434 (23).

The most detailed studies of the characteristics of LexA regulation have been performed with *E. coli* and show that the precise timing and duration of expression of each gene in the LexA regulon is determined by differential kinetics of LexA operator binding to the SOS gene promoters, namely that genes with high-affinity operators are induced late in the DNA damage response and that dissociation of LexA from its operators renders LexA prone for RecA*-

stimulated inactivation (19, 24, 25). Here we show that self-cleavage of the *A. baumannii* UmuDAb protein, which functions analogously to LexA (5–7), is also catalysed by RecA* preferentially when the regulator is in a DNA-unbound state (Fig. 4B). Thus, the affinity of UmuDAb for an individual operator sequence is likely to be an important factor in the timely response to DNA damage in *A. baumannii*. Similar binding kinetics of UmuDAb to the promoter regions of error-prone DNA polymerase V components, DdrR and itself suggest induction at the same time (Fig. 3D).

In bacteria, the development of quinolone resistance is a consequence of the mutagenic DNA damage response and is primarily driven by the acquisition of point mutations in DNA gyrase, and secondarily, by mutations in topoisomerase IV (26). Once *A. baumannii* strains have evolved to carry a quinolone-resistant gyrase allele, they exhibit a moderate level of quinolone resistance. It has been recently shown that in this intermediate resistance state, *A. baumannii* can enhance activation of the mutagenic DNA damage response and induce expression of prophage genes and phage genome multiplication (27). It is conceivable that in processes as diverse as the DNA damage response, the stepwise evolution of high quinolone resistance, and prophage induction are too complex to be regulated by a single transcription factor, albeit triggered by the same mechanism. The data presented here support the idea that the regulation of the DNA damage response across bacteria includes more than the archetype LexA/phage regulators/RecA (28, 29). We propose that small proteins, like gp7 and DdrR, are widespread in bacteria and act as accessory factors to regulatory proteins that undergo RecA*-mediated self-cleavage, like LexA, UmuDAb and phage regulators. Although they do not share sequence homology, these co-regulators have evolved to fine-tune a range of processes triggered by DNA damage, such as the activation of high- or low-fidelity DNA repair and the induction of prophages.

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Figures and figure legends

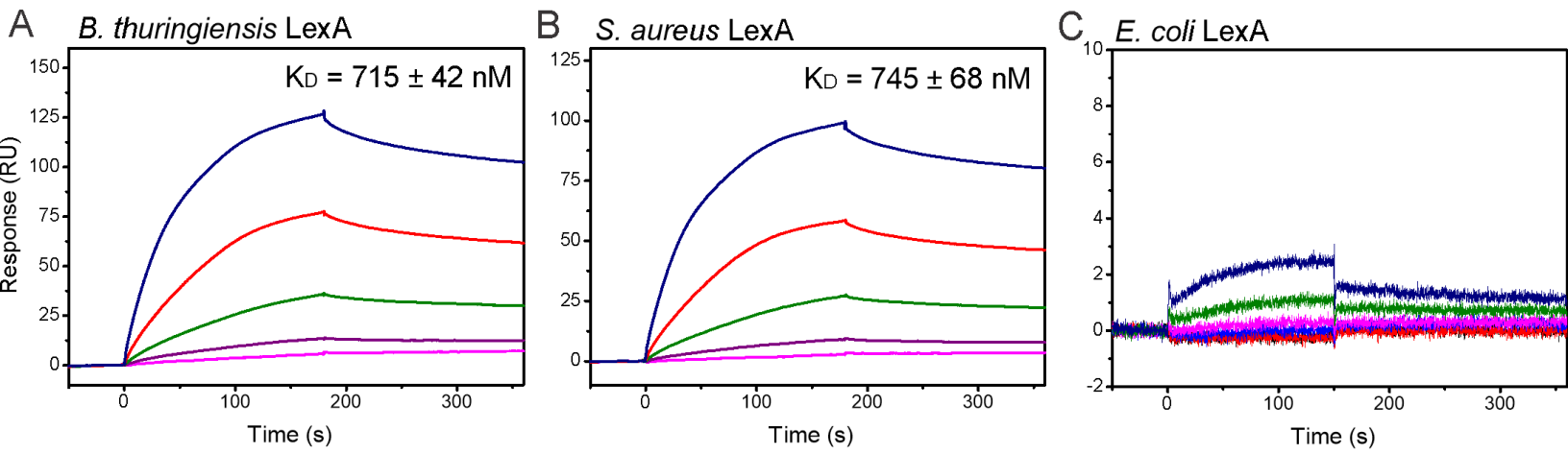
Figure 1: The DdrR protein binds LexA regulators from Gram-positive bacteria.
SPR sensorgrams of the interaction of DdrR with immobilized LexA protein from *B. thuringiensis* **(A)**, *S. aureus* **(B)** and *E. coli* **(C)**. DdrR was injected across the immobilized proteins at 3-fold dilutions (18-1458nM), for 180 s at 20 µL min^{-1}, followed by dissociation for 200 s. Apparent equilibrium dissociation constants (K_D) were determined by fitting the data in the dissociation phase (150 s after completion of sample injection) to the steady-state affinity model and are expressed as mean ± standard deviation of two titrations of DdrR for each immobilized protein.

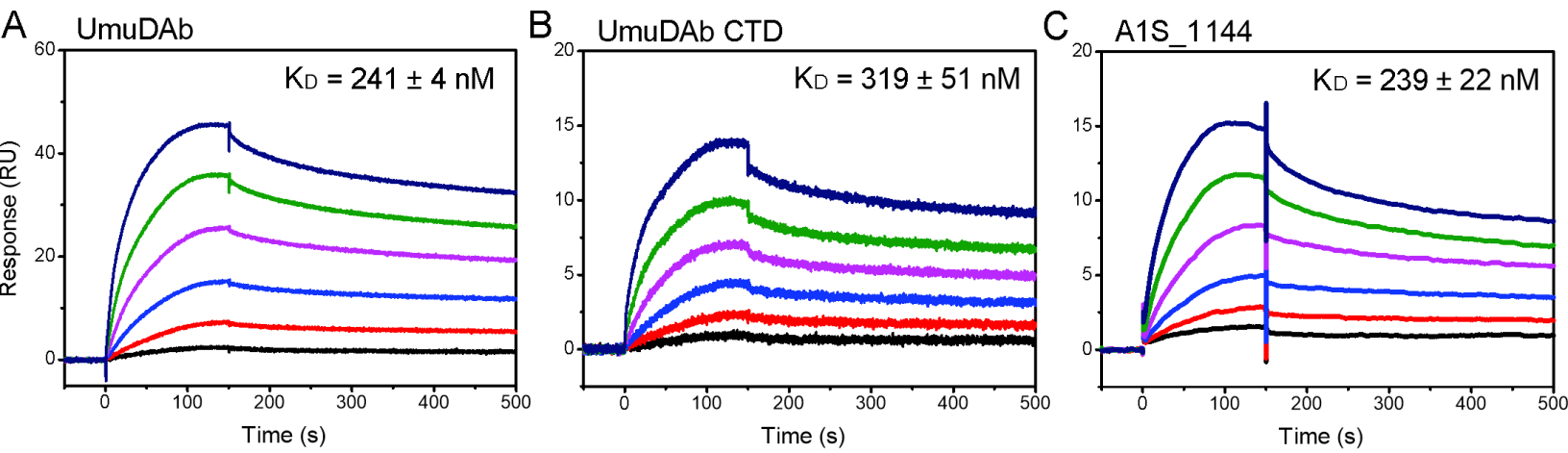
Figure 2: DdrR protein interacts with UmuDAb.
SPR sensorgrams of the interaction of DdrR with immobilized full-length UmuDAb **(A)**, the UmuDAb C-terminal domain (CTD) **(B)** and the putative phage repressor A1S_1144 **(C)**. DdrR was injected across the immobilized proteins at 2-fold dilutions (31.25-1000 nM), for 150 s at 20 µL min^{-1}, followed by dissociation for 360 s. Apparent equilibrium dissociation constants (K_D) were determined by fitting the data in the dissociation phase (150 s after completion of sample injection) to the steady-state affinity model and are expressed as mean ± standard deviation of two titrations of DdrR for each immobilized protein.

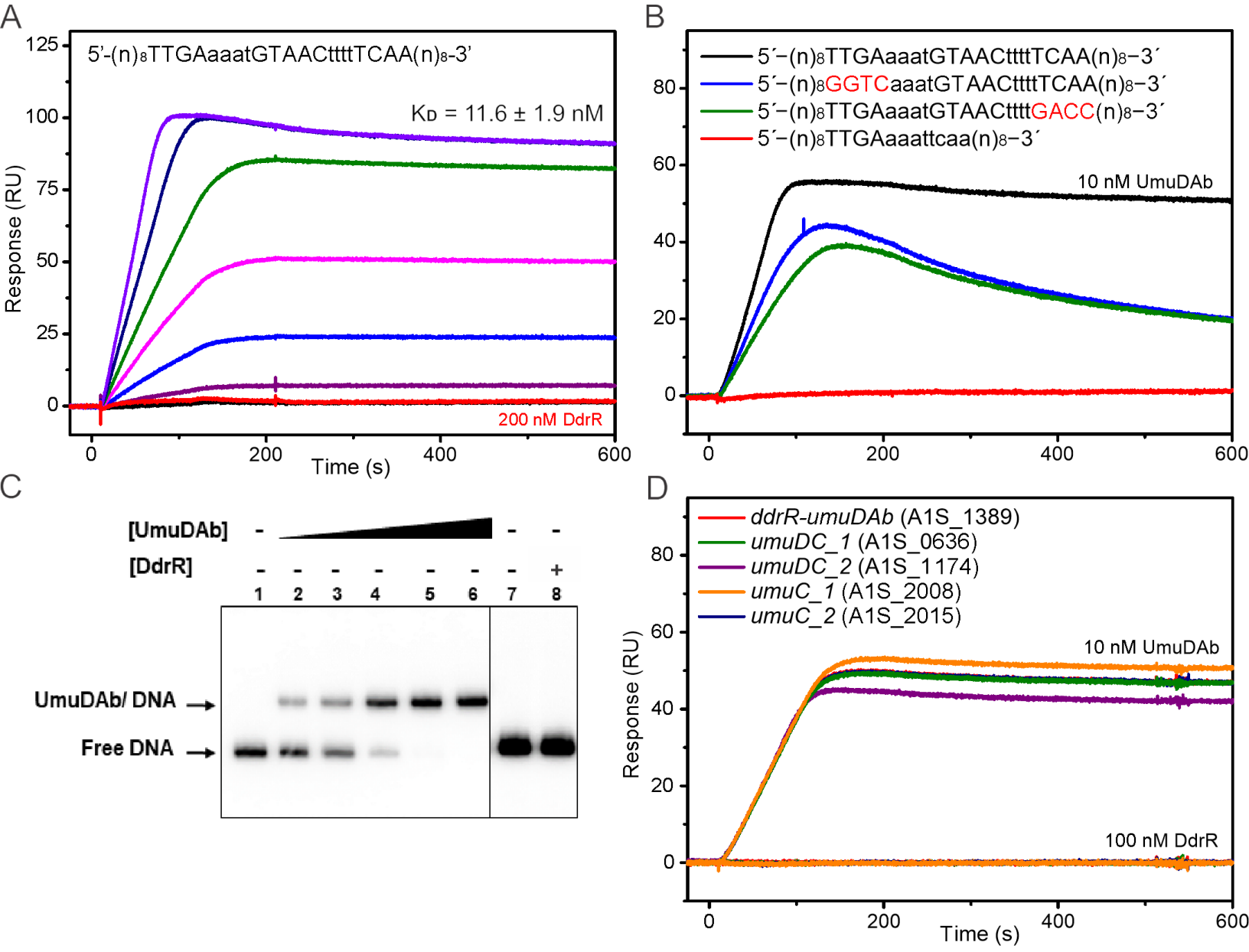
Figure 3: DdrR does not interact with the *umuDAb–ddrR* promoter region.
(A) SPR sensorgrams of UmuDAb injected across the chip-immobilized 37-bp DNA fragment, carrying the UmuDAb operator site found in the *ddrR-umuDAb* promoter region. UmuDAb was injected at the following concentrations: 2, 4, 6, 10, 12, 14, and 16 nM. The red line shows the absence of interaction between 200 nM DdrR and the UmuDAb operator. Proteins were injected over the immobilized DNA (~40 RU) for 210 s at 50 µL min^{-1}, followed by dissociation for 600 s. The sequence of the chip-immobilized UmuDAb operator fragment is presented above the sensorgram. The equilibrium dissociation constant (K_D) was determined by fitting the data in the dissociation phase at a time 150 s after the end of sample injection, to the steady-state affinity model and is shown with the standard deviation. Representative sensorgrams are shown for experiments performed in triplicate. **(B)** SPR sensorgrams of UmuDAb injected across the chip-

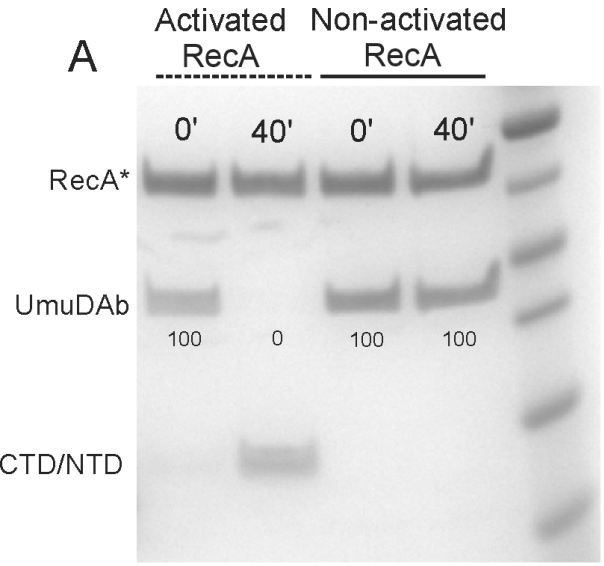
immobilized DNA fragments, carrying the wild-type UmuDAb target site found in the *ddrR-umuDAb* promoter region and three target sites with modified or shortened operator sequence. UmuDAb (10 nM) was injected over the immobilized DNA (~40 RU) for 210 s at 50 µL min^{-1}, followed by dissociation for 600 s. DNA sequences of the chip-immobilized fragments are shown for each corresponding colored curve. Representative sensorgrams are shown for experiments performed in duplicate. **(C)** EMSA of purified UmuDAb and DdrR protein binding to a 535 bp P^{32} end-labelled UmuDAb promoter fragment. The UmuDAb concentrations used in lanes 1 to 6 are 0 nM, 50 nM, 100 nM, 200 nM, 400 nM and 800 nM, respectively. The concentration of DdrR in lanes 1 to 7 is 0 nM and in lane 8, 400 nM. **(D)** SPR sensorgrams of 10 nM UmuDAb or 100 nM DdrR interacting with chip-immobilized 37-bp DNA fragments carrying the UmuDAb operator sequences of the four promoter regions of the polymerase V genes or the *ddrR-umuDAb* promoter region (Table S1) in their corresponding colors. Representative sensorgrams are shown for experiments performed in duplicate.

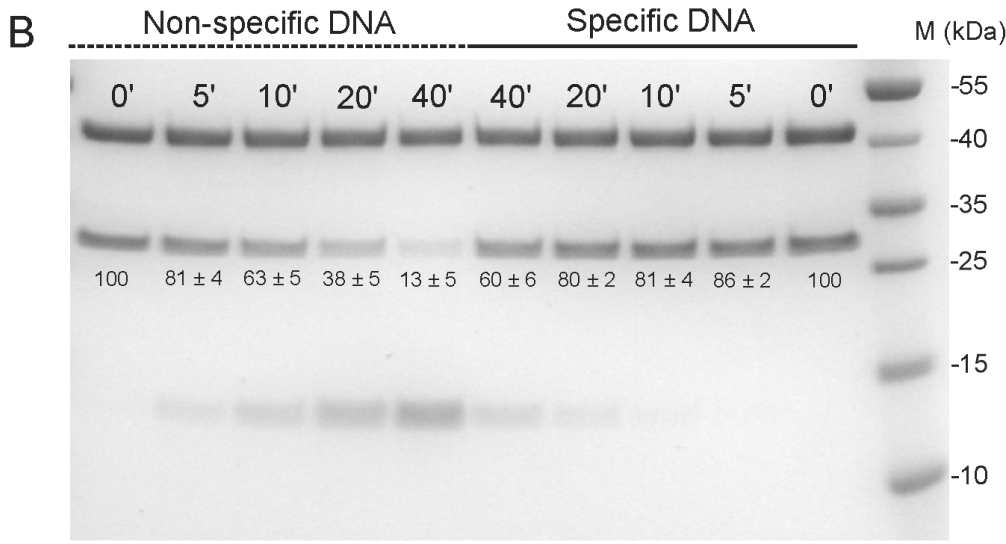
Figure 4: *A. baumanni* RecA* stimulates autolysis of the unbound UmuDAb.
Time course of UmuDAb self-cleavage in the presence of: (**A**) either activated (RecA*, RecA preincubated with ATP and ssDNA) or non-activated RecA; (**B**) RecA* and non-specific dsDNA fragment or a fragment encompassing the UmuDAb operator at a molar ratio of ~1:4 (UmuDAb monomer: DNA fragment) and (**C**) RecA* and DdrR's storage buffer or DdrR at a molar ratio of ~1:5 (UmuDAb monomer: DdrR monomer). Self-cleavage of UmuDAb was monitored using SDS-PAGE analysis of samples taken just before (0 min) or 40 min (panel A) or 5, 10, 20 and 40 min (panels B and C) after the addition of RecA* to the reaction mixtures. Intact UmuDAb monomer (UmuDAb), the C-terminal and N-terminal UmuDAb fragments (CTD and NTD, respectively), DdrR and RecA protein are indicated. Quantification of UmuDAb self-cleavage is shown below each band as the ratio (%) of the protein density value of samples from different time points relative to the density value of the initial sample (0 min), along with the standard error of the mean. Experiments were performed in duplicate and representative gels are shown.

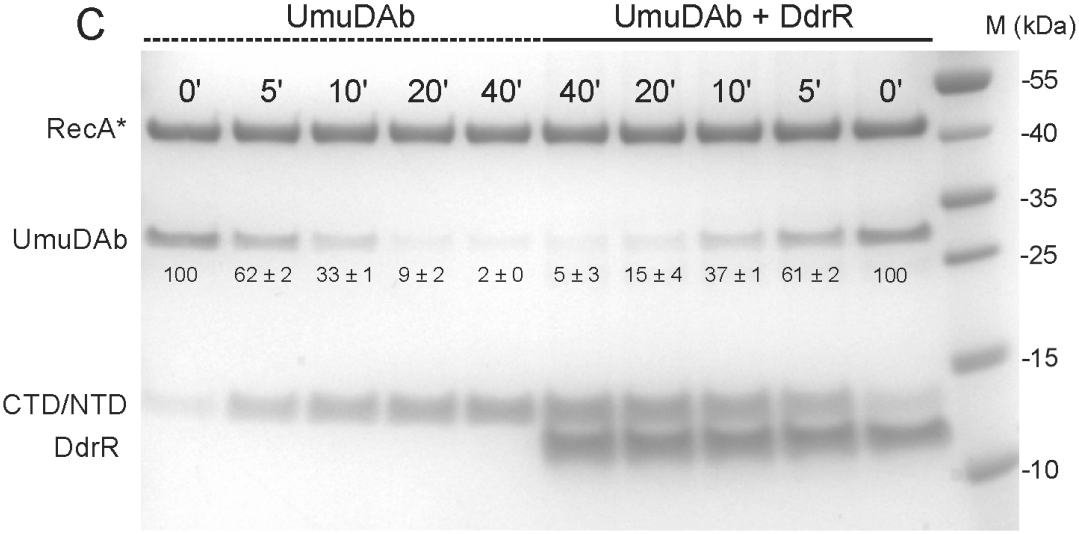

A
B. thuringiensis LexA
KD = 715 ± 42 nM
Response (RU)
Time (s)
B
S. aureus LexA
KD = 745 ± 68 nM
Time (s)
C
E. coli LexA
Time (s)


A
UmuDAb
KD = 241 ± 4 nM
Response (RU)
Time (s)
B
UmuDAb CTD
KD = 319 ± 51 nM
Time (s)
C
A1S_1144
KD = 239 ± 22 nM
Time (s)


A
5'-(n)8TTGAaaatGTAACttttTCAA(n)8-3'
KD = 11.6 ± 1.9 nM
200 nM DdrR
Response (RU)
Time (s)
B
5´−(n)8TTGAaaatGTAACttttTCAA(n)8−3´
5´−(n)8GGTCaaatGTAACttttTCAA(n)8−3´
5´−(n)8TTGAaaatGTAACttttGACC(n)8−3´
5´−(n)8TTGAaaattcaa(n)8−3´
10 nM UmuDAb
C
[UmuDAb]
[DdrR]
1 2 3 4 5 6 7 8
UmuDAb/ DNA
Free DNA
D
ddrR-umuDAb (A1S_1389)
umuDC_1 (A1S_0636)
umuDC_2 (A1S_1174)
umuC_1 (A1S_2008)
umuC_2 (A1S_2015)
10 nM UmuDAb
100 nM DdrR
Response (RU)
Time (s)


A
Activated RecA
Non-activated RecA
0'
40'
0'
40'
RecA*
UmuDAb
100
0
100
100
CTD/NTD


B
Non-specific DNA
Specific DNA
M (kDa)
0'
5'
10'
20'
40'
40'
20'
10'
5'
0'
100
81 ± 4
63 ± 5
38 ± 5
13 ± 5
60 ± 6
80 ± 2
81 ± 4
86 ± 2
100
-55
-40
-35
-25
-15
-10


C
UmuDAb
UmuDAb + DdrR
M (kDa)
0'
5'
10'
20'
40'
40'
20'
10'
5'
0'
RecA*
UmuDAb
100
62 ± 2
33 ± 1
9 ± 2
2 ± 0
5 ± 3
15 ± 4
37 ± 1
61 ± 2
100
CTD/NTD
DdrR
-55
-40
-35
-25
-15
-10