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The *Acinetobacter* regulatory UmuDAb protein cleaves in response to DNA damage with chimeric LexA/UmuD characteristics

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Abstract

In the DNA damage response of most bacteria, UmuD forms part of the error-prone (UmuD′$_2$)C polymerase V, and is activated for this function by self-cleavage after DNA damage. However, the *umuD* homolog (*umuDAb*) present throughout the *Acinetobacter* genus encodes an extra N-terminal region, and in *A. baylyi,* regulates transcription of DNA-damage induced genes. UmuDAb expressed in cells was correspondingly larger (24 kDa) than the *Escherichia coli* UmuD (15 kDa). DNA damage from mitomycin C or UV exposure caused UmuDAb cleavage in both *E. coli* wild type and Δ*umuD* cells on a timescale resembling UmuD, but did not require UmuD. Like the self-cleaving serine proteases LexA and UmuD, UmuDAb required RecA for cleavage. This cleavage produced a UmuDAb′ fragment of a size consistent with the predicted cleavage site of Ala83-Gly84. Site-directed mutations at Ala83 abolished cleavage, as did mutations at either the Ser119 or Lys156 predicted enzymatic residues. Co-expression of the cleavage site mutant and an enzymatic mutant did not allow cleavage, demonstrating a strictly intramolecular mechanism of cleavage that more closely resembles the LexA-type repressors than UmuD. These data show that UmuDAb undergoes a post-translational, LexA-like cleavage event after DNA damage, possibly to achieve its regulatory action.

Keywords

DNA damage; serine protease; UmuD; LexA; SOS response

Introduction

DNA damaged in *Escherichia coli* and other bacteria by UV light, mitomycin C (MMC), or antibiotics results in the induction of many genes, termed SOS genes, that carry out error-free repair (e.g. *polB, recA, recN, sulA, uvrB,* and *uvrD*) (Friedberg, 1995) and error-prone repair of damaged DNA (*umuD*, *umuC*, and *dinB/P*) (Little & Mount, 1982; Walker, 1984). This induction begins when an abundance of ssDNA induces formation of RecA*, which is the form of RecA that promotes the proteolytic self-cleavage of the LexA repressor (Horii *et al*., 1981). LexA negatively regulates SOS gene transcription (Brent & Ptashne, 1981; Mount *et al*., 1972) by binding to a 20 nucleotide "SOS box" (Lewis *et al*., 1992) in SOS gene promoters, but LexA self-cleavage induces the expression of SOS genes after DNA damage.

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The error prone SOS response requires the SOS genes *umuDC* and *recA*. The 15 kDa UmuD protein forms homodimers, and when bound to UmuC, functions as a checkpoint in delaying cell division, allowing time for error-free repair mechanisms to act (Opperman *et al.*, 1999). RecA*, besides assisting in LexA self-cleavage, also facilitates the intermolecular self-cleavage of $UmuD_2$ (Burckhardt *et al.*, 1988; Nohmi *et al.*, 1988; Shinagawa *et al.*, 1988). Cleaved $UmuD'_2$ bound to UmuC (Woodgate *et al.*, 1989) forms DNA polymerase V about 20–40 minutes after DNA damage (Sommer *et al.*, 1998). Pol V carries out trans-lesion replication of damaged DNA, but lacks 3′–5′ exonuclease activity and thus is error-prone (Tang *et al.*, 1999), resulting in SOS mutagenesis.

Research in non-*E. coli* species reveals variation in LexA function and number, as well as different SOS genes and SOS boxes bound by LexA. In *Acinetobacter baylyi* strain ADP1, additional differences also exist. In ADP1, *recA* (Rauch *et al.*, 1996) and *ddrR* (a gene of unknown function that is unique to the *Acinetobacter* genus; Hare *et al.*, 2006, Hare *et al.*, 2012) are induced after DNA damage but only *ddrR* requires RecA for induction (Whitworth, 2000). The ADP1 *recA* and *ddrR* promoters also lack a known or predicted SOS box (Gregg-Jolly & Ornston, 1994; Hare *et al.*, 2006). Additionally, typical DNA damage response genes encoding LexA, SulA, or sigma factor σ^{38} are not found in *A. baylyi* or *A. baumannii* (Hare *et al.*, 2006; Robinson *et al.*, 2010), and accordingly, SOS mutagenesis has not been observed in *Acinetobacter* (Berenstein, 1987) with the notable exception of the emerging pathogens *A. baumannii* and *A. ursingii* (Hare *et al.*, 2012).

Further differences are centered on the *umuDC* operon in *Acinetobacter*. In ADP1, *A. baumannii*, and seven other *Acinetobacter* species examined, the *umuD* homolog (termed *umuDAb*; Hare *et al.,* 2012) encodes an extra 59-aa N-terminus region relative to the typical bacterial *umuD*, and is always located adjacent to *ddrR*. Conversely, *umuDC* operons similar in size to those found in *E. coli* are present in only 50% of *Acinetobacter* species studied, seemingly acquired through horizontal gene transfer (Hare *et al.*, 2012). Also unlike typical UmuD function, this newly described *umuDAb* allele regulates transcription of the adjacent DNA damage-induced *ddrR* gene (Hare *et al.*, 2006), as well other genes (J. M. Hare and J. A. Bradley, unpublished) in ADP1.

This *Acinetobacter* UmuDAb possesses both the conserved serine-lysine catalytic dyad required by UmuD, LexA, and some bacteriophage repressors for self-cleavage (Paetzel *et al.*, 1997; Walker, 2001) as well as the (Ala/Cys)-Gly cleavage site (Hare *et al.*, 2006; Hare *et al.*, 2012), which suggests that UmuDAb may self-cleave by a similar mechanism. The regulatory activity and possession of an N-terminal domain (Hare *et al.*, 2006) that both UmuDAb and LexA possess further predict that UmuDAb may conduct intramolecular cleavage like LexA, instead of the intermolecular cleavage of $UmuD_2$ (McDonald *et al.*, 1998) that is required for its participation in SOS mutagenesis. However, UmuDAb is more similar to UmuD and its homologs (42–46% amino acid identity) than to LexA, whose size it shares. UmuDAb shares only 37% identity with LexA, and this similarity is restricted to the self-cleaving carboxy-terminus, not the DNA-binding N-terminal domain of LexA (Figure 1). Because ADP1 possesses a mutated *umuC* gene (Hare *et al.*, 2006), and the *Acinetobacter* species capable of DNA damage-induced mutagenesis possess both *umuDC* and *umuDAb* genes (Hare *et al.*, 2012), the ability of UmuDAb to participate in SOS mutagenesis is unknown.

The unexpected observation that a homolog of the error-prone polymerase accessory, UmuD, regulates genes in response to DNA damage highlights the need to determine a mechanism that ties UmuDAb action to the DNA damage response. We hypothesize that UmuDAb responds to DNA damage with self-cleavage. Determining whether UmuDAb self-cleaves in response to DNA damage, and by what mechanism, will help elucidate the

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function of UmuDAb in the *Acinetobacter* DNA damage response as regulator and/or polymerase accessory.

Materials and Methods

Bacterial Strains and Plasmids

The *Escherichia coli* strains used, and their genotypes relevant to this study, were AB1157 (wild type), 315 (AB1157 Δ*umuD772::kan*), AB2463 (AB1157 *recA13*), and DH5α (*recA1*). Both *recA*$^-$ alleles, which are missense point mutations at G160D (*recA1*) or L51F (*recA13*), are defective for all activities except ssDNA binding (Lauder & Kowalczykowski, 1993).

QIAGEN's EasyXpress Protein Synthesis PCR process was used to amplify *umuDAb* from plasmid pJH1, which contains *umuDAb* in its native chromosomal context (Hare *et al*., 2006). The *umuDAb* PCR product was cloned into *Xba*I and *Bam*HI restriction sites of the Qiagen EasyExpress pIX3.0 vector to form plasmid pIX2.

pIX2AtoY, pIX2GtoE, pIX2StoA, and pIX2KtoA resulted from site-directed mutagenesis of the *umuDAb* codons for A83, G84, S119, or K156 in pIX2 with the Stratagene QuikChange II kit. These mutations were confirmed by double-stranded DNA sequencing of the plasmids. Descriptions of these strains and plasmids are in Table 1.

Cell extract preparation

Total protein cellular lysates were prepared starting with overnight cultures grown shaking in 3 mL of LB broth with ampicillin at 37°C. Cultures were diluted 1:10 in LB plus ampicillin and grown shaking for an additional 3 hours at 37°C to enter early exponential phase. After 3 hours, the culture was split in half, with 2 μg mL^{-1} MMC added to one culture. Alternately, for UV treatment, 400 μL of cell culture was washed and resuspended in phosphate-buffered saline, put in a 5.3 cm diameter watch-glass, and exposed to 200 J m^{-2} UV-C light (or a mock treatment), using a Stratagene UV Stratalinker in the dark. These UV-exposed samples were pelleted and resuspended in media containing 100 μg mL^{-1} ampicillin. Following DNA damage or a mock treatment, cultures were grown for additional time ranging from 5 – 60 minutes before samples were collected. Samples were pelleted and resuspended in Laemmli buffer containing 5% 2-mercaptoethanol, and stored at −20 °C.

Immunoblot and protein analysis

Proteins were separated on 4–20% Tris-HCl SDS-PAGE TGXgels in running buffer (25 mM Tris base, 192 mM glycine, 10% SDS). Frozen lysates were boiled for 5 minutes and held on ice for 5 minutes before use. The RC DC Protein Assay was performed to equalize the amount of total protein loaded in each lane. All protein supplies were obtained from Bio-Rad unless otherwise stated.

Proteins were transferred to an Immun-Blot PVDF membrane using a Trans-Blot apparatus. The membrane was blocked overnight at 4°C in 0.05% Tween-20 in Tris buffered saline (TBS) containing 5% non-fat dry milk on a Belly Dancer. Primary antibodies used at 1:10,000 dilutions were either an anti-peptide antibodies directed against amino acids 5–19 of UmuDAb, or polyclonal antibody prepared by GenScript by injection of purified UmuDAb (produced by GenScript) into rabbits and purified by protein A chromatography. Goat anti-rabbit HRP-conjugated secondary antibody was used at a dilution of 1:32,000. All antibody incubations were carried out for 1 hour in 0.05% TBS Tween-20 in 2.5% milk on a Belly Dancer. Precision StrepTactin-HRP Conjugate was added with the secondary antibody

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to visualize the protein size marker (Precision Plus Protein WesternC Standards). The membrane was washed five times (10 minutes each) with 0.01% TBS-Tween 20 after each antibody incubation. SuperSignal West Pico chemiluminescent substrate (Pierce) was used to visualize proteins after exposure to X-ray film.

Results and Discussion

UmuDAb is larger than UmuD and is expressed from its native promoter in *E. coli*

UmuDAb expression and cleavage was investigated after transforming *E. coli* AB1157 wild-type and mutant cells with plasmids bearing various *umuDAb* alleles. This allowed us to test the effects of *recA* and *umuD* mutations on UmuDAb cleavage in a context of the otherwise intact and well-studied DNA damage response of *E. coli. E. coli* cells were exposed to DNA damaging agents and immunoblot analyses of cell lysates were performed with anti-UmuDAb peptide or polyclonal antibodies.

To test whether the *umuDAb* ORF truly encoded an extra-large UmuDAb protein, plasmid pJH1, which contains 2.2 kbp of DNA from ADP1, including *umuDAb* in its native chromosomal context, was used as a UmuDAb expression source. This approach was feasible because *Acinetobacter* promoters are typically highly expressed in *E. coli* (Shanley *et al.*, 1986). Lysates from *E. coli* wild type and Δ*umuD* cells, carrying pJH1 but not treated with MMC expressed a ~24 kDa protein (Figure 2), consistent with the predicted molecular weight of 23.4 kDa , and demonstrating that the protein encoded by *umuDAb* was indeed larger than the 15 kDa UmuD (Kitagawa *et al.*, 1985). This protein was not expressed in cells containing only the pUC19 vector of pJH1. This UmuDAb expression in uninduced *E. coli* may be due to the lack of an *E. coli* SOS box in the *umuDAb* promoter, although transcription of *umuDAb* was also observed in uninduced ADP1 cells (Hare *et al.*, 2006).

UmuDAb disappears from MMC- and UV-treated cells with UmuD-like timing, but independently of *E. coli* UmuD

The *umuDAb* ORF was then sub-cloned into the vector pIX3.0 to form pIX2, which was used for the majority of the experiments because it expressed the 24 kDa UmuDAb (Figure 2), but did not contain ADP1 chromosomal DNA surrounding *umuDAb* as a potential confounding factor. To test whether DNA damage could cause UmuDAb cleavage, wild type *E. coli* cells carrying either pJH1 or pIX2 were grown to log phase and treated with a dose of MMC (2 ug mL^{-1}) that is sufficient to induce the SOS response in *E. coli* (Moreau, 1987) and the transcription of *ddrR* (Hare et al., 2006) and *recA* (Rauch et al., 1996) in *Acinetobacter*. UmuDAb was not detected after one hour of MMC treatment (Figure 2A, B).

To compare the timing of this UmuDAb disappearance to the self-cleaving UmuD and LexA proteins, ImageJ Software (National Institutes of Health) was used to determine the percent of UmuDAb remaining at specific times after DNA damage. The 24 kDa UmuDAb band expressed from either plasmid disappeared from MMC-treated cell lysates in a time-dependent manner, whereas the amount of UmuDAb was unchanged over time in non-MMC treated cells (Fig. 3A, B). A cross-reacting band of ~19 kDa expressed in the vector control (Fig 3A, lane 1; Fig. 3B, lane 2) also was unchanged. By forty-five minutes post-MMC treatment, virtually all of the UmuDAb had disappeared. Based on Figure 3 and additional experiments, the half-life of UmuDAb after MMC treatment was estimated to be ~20 minutes, which is similar to the ~ 20 minute half-life observed for UmuD after UV exposure (Opperman *et al.*, 1999) but longer than the <5 minute half-life for LexA after either UV or MMC treatment (Sassanfar & Roberts, 1990). After nalidixic acid treatment, UmuD also persists in an uncleaved form longer (~60 minutes) than LexA (~5 minutes) (Mustard & Little, 2000).

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UmuDAb expression and cleavage was also examined in Δ*umuD* cells to test whether *E. coli* UmuD was required for UmuDAb disappearance. The 46% identity in the C-terminal dimerization domains of UmuD and UmuDAb suggested that UmuD-UmuDAb heterodimerization might allow UmuD to intermolecularly cleave UmuDAb, which might itself have no inherent self-cleavage ability. However, we observed UmuDAb to be expressed and disappear with similar timing in Δ*umuD* cells as in wild type *E. coli* (Figures 2, 3), demonstrating that *E. coli* UmuD is not required for UmuDAb expression from its native promoter, nor its disappearance after DNA damage through intermolecular interactions with *E. coli* UmuD.

If UmuDAb cleavage were responding to DNA damage like LexA and UmuD, one would expect cleavage to result from treatment with other DNA damaging agents. Cells carrying the pIX2 plasmid were exposed to UV-C in amounts sufficient to induce UV-mutagenesis in *E. coli* as well as *Acinetobacter* (Hare *et al.*, 2012), which caused the disappearance of UmuDAb (Figure 3C), suggesting that UmuDAb cleavage was in response to DNA damage in general, and not a specific response to MMC.

UmuDAb cleavage requires *recA*

In *E. coli,* RecA is activated by DNA damage to subsequently bind to, and facilitate the self-cleavage of, both UmuD and LexA (Shinagawa *et al.*, 1988). UmuDAb disappearance was examined in *recA*$^-$ *E. coli* strains to test the hypothesis that RecA is similarly required for UmuDAb cleavage. As predicted, in both DH5α *recA1* cells as well as the *recA13* strain of AB1157 (AB2463) (Howard-Flanders & Theriot, 1966), UmuDAb expressed from either pJH1 or pIX2 did not disappear after 1 hour of MMC treatment (Figure 4) or UV exposure (data not shown). This absolute requirement for RecA in UmuDAb disappearance after DNA damage suggests that the disappearance results from cleavage, not general degradation, and is consistent with studies of LexA and UmuD self-cleavage.

Site-directed mutagenesis of predicted cleavage or active site residues abolish cleavage

Cleavage site mutants (CSM) of *E. coli* UmuD of C24D/G25D (McDonald *et al.*, 1998), G25E or C24Y (Nohmi *et al.*, 1988) severely reduced SOS mutagenesis, as did active site mutants (ASM) S60A or K97A in the serine and lysine residues required for nucleophilic attack on the cleavage site (Nohmi *et al.*, 1988). Similar mutations in LexA, e.g. S119A or K156A, abolished LexA self-cleavage (Slilaty & Little, 1987). Because most UmuD mutations that impair SOS mutagenesis act by interfering with cleavage (Koch *et al.*, 1992), we hypothesized that similar UmuDAb CSM and ASMs would prevent UmuDAb cleavage.

To test whether UmuDAb cleavage occurred at the A83-G84 cleavage site predicted by alignment with other UmuD proteins (Figure 1 and Hare *et al*, 2006), two CSMs were constructed by site-directed mutagenesis of pIX2. The G84E mutation had minimal effect on UmuDAb cleavage (data not shown), but the A83Y mutation completely abolished cleavage after MMC (Figure 5A) or UV treatment (data not shown). Such variation in effect was also observed for UmuD CSMs (McDonald *et al.*, 1998; Nohmi *et al.*, 1988). UmuDAb ASMs S119A or K156A also abolished cleavage in both wild type and Δ*umuD E. coli* cells after MMC (Figure 5A) or UV treatment (data not shown). These multiple, independent observations of cleavage impairment suggests that UmuDAb “disappearance” is self-cleavage at the A83-G84 site, requiring functional residues S119 and K156 in a reaction similar that used by LexA and UmuD, and not due to plasmid-based overexpression.

UmuDAb cleavage is strictly intramolecular

The observation that UmuDAb cleavage did not require *E. coli* UmuD did not preclude UmuDAb self-cleavage occurring by a UmuD-like intermolecular mechanism. The use of

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polyclonal antibodies directed against purified UmuDAb allowed us to visualize UmuDAb cleavage products and thus test whether UmuDAb disappearance after DNA damage was truly cleavage at the A83-G84 site, and also whether UmuDAb cleavage was inter- or intramolecular. In AB1157 and Δ*umuD* (pACYC2) cell extracts, we observed a ~14 kDa UmuDAb′ cleavage product appearing in MMC treated cells (Fig. 5B, C and multiple other experiments not shown), which was consistent with the predicted UmuDAb A83-G84 cleavage site shown in Figure 1 (Hare *et al.*, 2006).

Complementation experiments in *E. coli*, where co-expressed UmuD CSM and ASM mutants rescued cleavage, established an intermolecular mechanism of UmuD self-cleavage (McDonald *et al.*, 1998). We constructed Δ*umuD* strains expressing multiple forms of UmuDAb from pACYC184 and pIX3.0 vectors to conduct similar investigations of UmuDAb cleavage. Controls confirmed WT UmuDAb cleavage, and uncleavable UmuDAb A83Y (CSM) and UmuDAb S119A (ASM1) after MMC treatment, when expressed in Δ*umuD* cells from pACYC184 (Figure 5B, lanes 2–7). However, in four independent attempts at complementation where UmuDAb A83Y (CSM) and either UmuDAb S119A (ASM1) or UmuDAb K156A (ASM2) were co-expressed in Δ*umuD* cells, no UmuDAb′ cleavage products were observed (Figure 5B, lanes 8–11 and 5C, lanes 7, 8), regardless of which plasmid drove CSM or ASM expression. This lack of complementation of CSM and ASM action indicated a strictly intramolecular mechanism of cleavage for UmuDAb, although improper folding of these mutants could not be ruled out as a cause of these results. When wild type UmuDAb was co-expressed in Δ*umuD* cells with either a CSM or ASM (Figure 5B, lanes 12–15; 5C, lanes 3–6), as a control, UmuDAb′ cleavage products were observed, indicating cleavage competence of UmuDAb in cells expressing multiple UmuDAb forms.

In *E. coli*, UmuD forms dimers that cleaves intermolecularly (McDonald *et al.*, 1998), although recent evidence shows that *E. coli* UmuD can cleave intramolecularly, albeit only when a specific mutation is engineered into UmuD to prevent homodimerization (Ollivierre *et al.*, 2011). However, we found that UmuDAb, unlike UmuD, does not cleave intermolecularly, although UmuDAb contains the conserved asparagine required for UmuD dimerization (Ollivierre *et al.*, 2011). In this respect, UmuDAb naturally behaves like a monomer, although its homology to other self-cleaving serine proteases supports the hypothesis that it may dimerize. This intramolecular cleavage of UmuDAb, as well as its previously observed regulatory action and amino acid motifs (Hare *et al.*, 2006) thus more resembles a LexA- or bacteriophage-like repressor action than UmuD polymerase accessory function. However, there is no similarity between the DNA-binding N-terminal domain of LexA and UmuDAb (Figure 1), which may indicate an indirect mechanism of UmuDAb transcriptional regulation. UmuD belongs to the class of intrinsically disordered proteins that regulate cell processes through different interactions with a variety of partners such as DNA Pol III, the error-prone polymerases DinB and UmuC, as well as RecA and the beta-sliding clamp (Simon *et al.*, 2008). UmuDAb regulatory action might result from interaction with yet an additional partner, to yield the novel function of this UmuD-like protein. These characteristics of UmuDAb action in the DNA damage response of *Acinetobacter* reveal the various ways that cells can respond to DNA damage.

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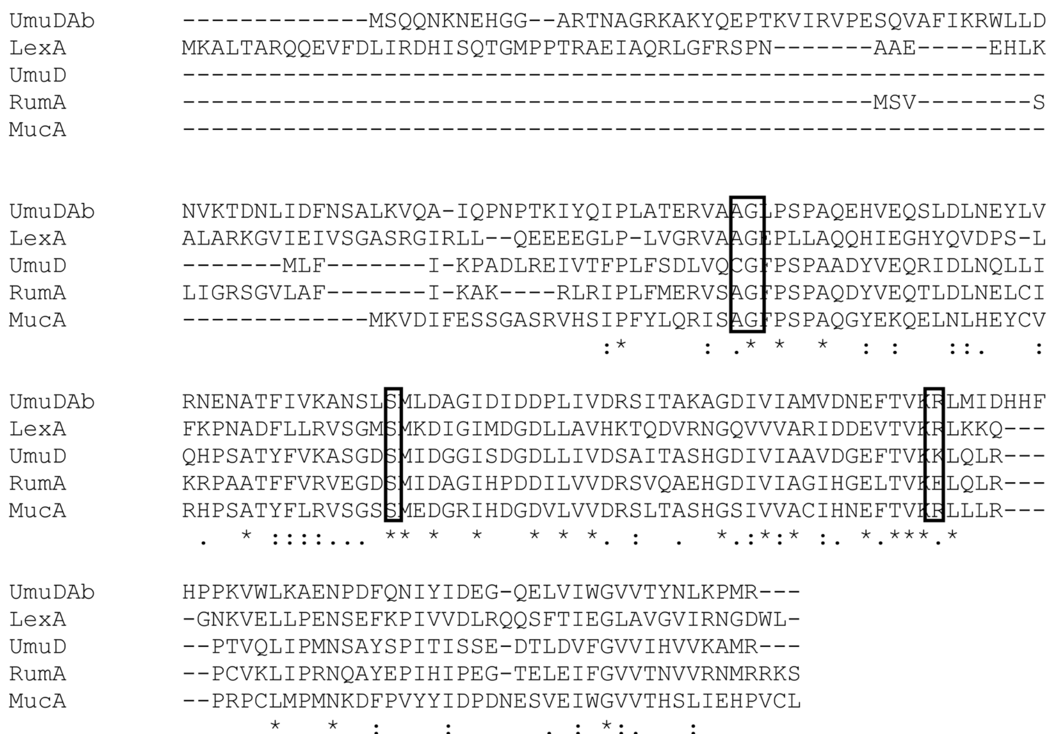

Figure 1.

Multiple alignment of UmuDAb with LexA, UmuD and UmuD homologs RumA and MucA shows lack of conservation of N-terminal region of UmuDAb to LexA. UmuDAb is more similar to UmuD and its homologs than LexA throughout its sequence. Clustal Omega (Sievers *et al.*, 2011) was used to create the multiple alignment of ADP1 UmuDAb, *E. coli* LexA and UmuD, RumA from *Providencia rettgeri* IncJ R391 plasmid, and MucA from *Salmonella typhimurium* IncN plasmid R46/pKM101. Asterisks indicate 100% conserved residues; colons indicate functionally conserved residues. The A/C-G cleavage site and serine and lysine active site residues are boxed.

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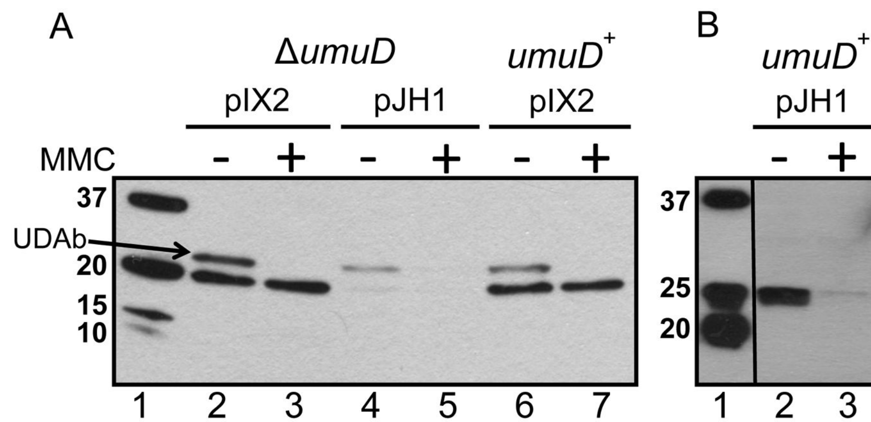

Figure 2.

UmuDAb is larger (~24 kDa) than the *E. coli* UmuD protein, expressed in *E. coli* cell lysates constitutively from its native promoter on pJH1, or expression vector on pIX2, and disappears after MMC treatment. Representative immunoblots of *E. coli* cell lysates collected 1 hour after mock (–) or 2 μg mL^{-1} treatment (+) with MMC are shown, probed with anti-peptide antibody against the N-terminus of UmuDAb. UmuDAb is designated by the "UDAb" arrow. A cross-reacting band of ~19 kDa does not disappear with MMC treatment, and was used as a protein loading control. Molecular mass size markers in lanes A1 and B1 are shown in kDa. (A) Lanes 2–5, Δ*umuD* strain 315 carrying either plasmid pIX2 (lanes 2, 3) or pJH1 (lanes 4, 5). Lanes 6 and 7 contain wild type AB1157 carrying pIX2. (B) Lanes 2 and 3 contain AB1157 carrying pJH1.

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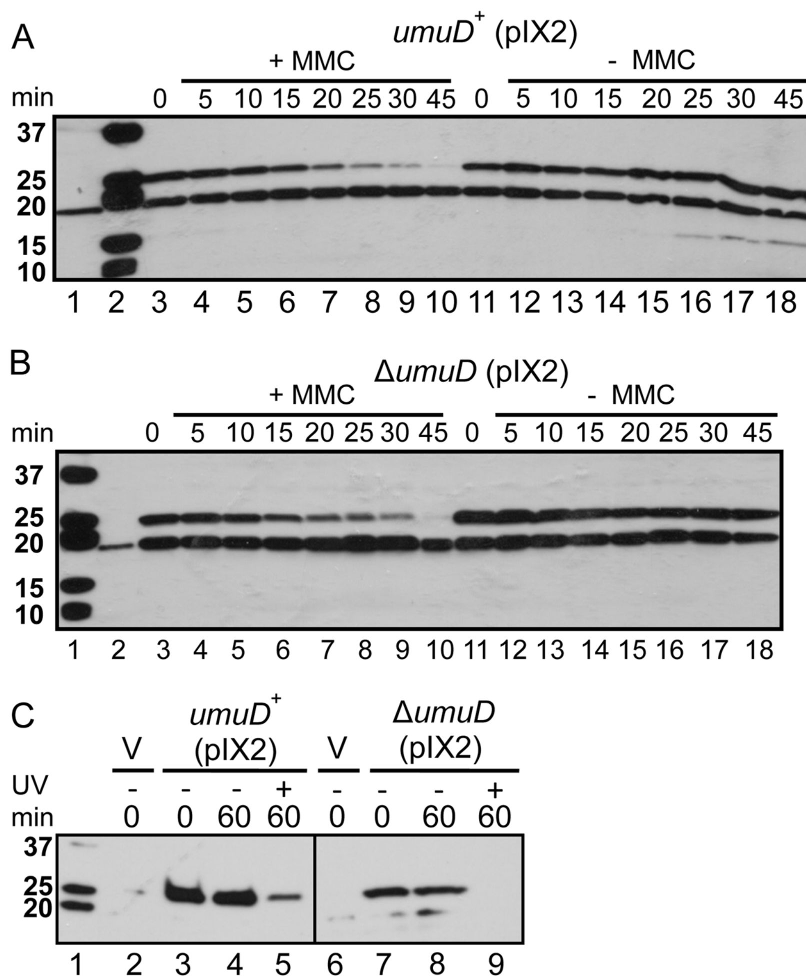

Figure 3.

UmuDAb disappears over time in both wild type and Δ*umuD E. coli* cells after DNA damage mediated by either mitomycin C or UV. Immunoblots of cell lysates either untreated (−), or treated with 2 μg mL^{-1} MMC or 200 J cm^{-2} UV-C light (+), were probed with anti-peptide antibody against the N-terminus of UmuDAb. Molecular mass size markers in lanes A2, B1 and C1 are shown in kDa. A cross-reacting band of ~19 kDa does not disappear with MMC treatment, and was used as a protein loading control. (A) AB1157 (pIX2) or (B) Δ*umuD* (pIX2) cell lysates were collected over time after MMC addition (lanes 3–10) or mock MMC treatment (lanes 11–18). Lanes A1 and B2 contain AB1157 (pIX3.0) and Δ*umuD* (pIX3.0) cell lysates, respectively, as vector controls. (C) UV treatment also induces UmuDAb disappearance in AB1157 (pIX2) (lanes 3–5) or Δ*umuD* (pIX2) (lanes 7–9) cell lysates. Lanes 2 and 6 contain AB1157 (pIX3.0) and Δ*umuD* (pIX3.0) cell lysates respectively, as vector controls.

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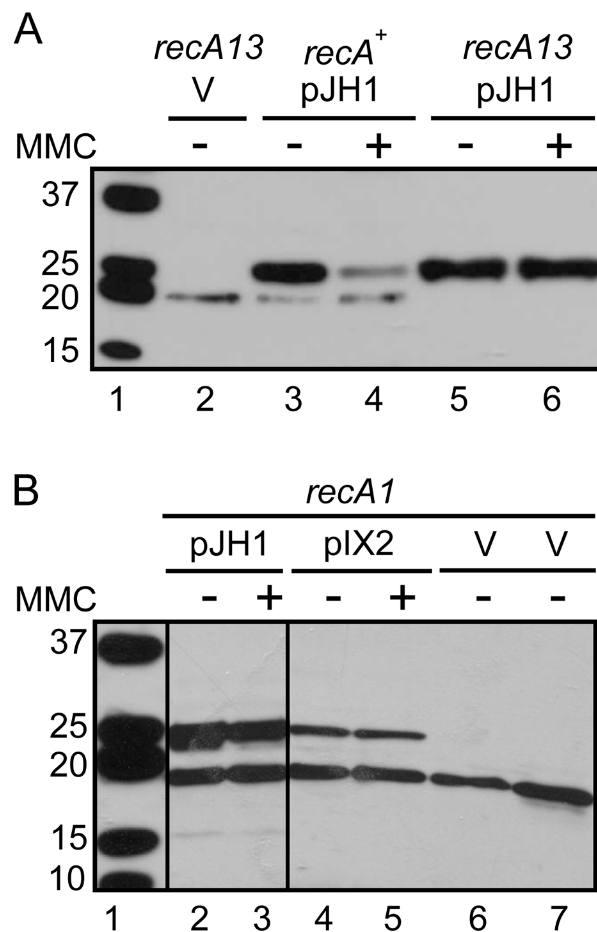

Figure 4.

RecA is required for UmuDAb cleavage. UmuDAb expressed in cell lysates of (A) AB2463 *recA13* or (B) DH5α *recA1* cells in the absence of MMC (–) does not disappear after one hour of 2 μg mL^{-1} MMC treatment (+). Immunoblots of cell lysates were probed with anti-peptide antibody against the N-terminus of UmuDAb. Molecular mass size markers in lanes A1 and B1 are shown in kDa. A cross-reacting band of ~19 kDa is seen and does not disappear after MMC treatment. Cell lysates were collected from: (A) Lane 2, AB2463 *recA13* (pUC19) vector control; Lanes 3, 4 AB1157 (pJH1); Lanes 5, 6 AB2463 *recA13* (pJH1). (B) Lanes 2, 3 DH5α (pJH1); Lanes 4, 5 DH5α (pIX2); Lane 6, AB1157 (pUC19) vector control for lanes 2, 3; Lane 7, AB1157 (pIX-3.0) vector control for lanes 4, 5. A cross-reacting band of ~19 kDa does not disappear with MMC treatment, and was used as a protein loading control.

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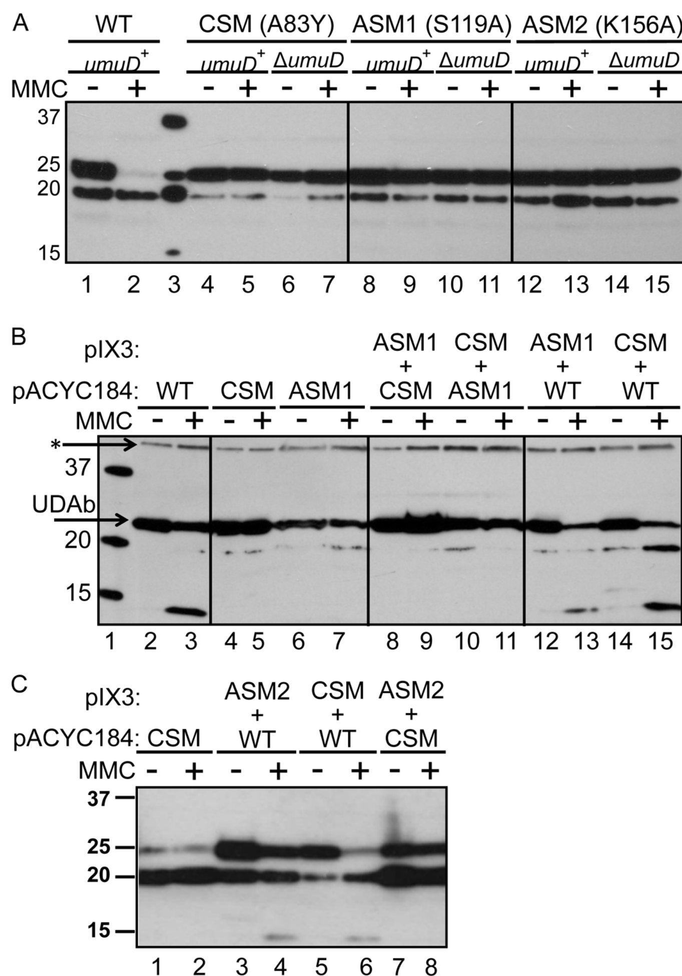

Figure 5.

Absence of UmuDAb cleavage in active site mutants (ASM) or cleavage site mutants (CSM) demonstrates requirement for predicted active site and cleavage site residues, as well as an intramolecular cleavage mechanism. Cells were either exposed to 0 MMC (–) or 2 μg mL^{-1} MMC (+) for one hour. An immunoblot of *E. coli* cell lysates, representative of an experiment performed 3–4 times, is shown for each panel. Molecular mass size markers in lanes A3, B1, and designated by lines in panel C are shown in kDa. Cross-reacting bands of ~19 kDa (and ~50 kDa, panel B) do not disappear after MMC treatment and were used as protein loading controls. "pIX3:" and "pACYC184:" refer to the plasmid vector used to express the particular UmuDAb protein. (A) Wild type UmuDAb (WT) expressed from pIX2 is cleaved in AB1157 wild type *umuD*+ cells, but mutant proteins UmuDAb A83Y (CSM), lanes 4–7; UmuDAb S119A (ASM1), lanes 8–11; and UmuDAb K156A (ASM2), lanes 12–15; (expressed from pIX2AtoY, pIX2StoA, and pIX2KtoA, respectively), are uncleaved after exposure to MMC, in both *umuD*+ and Δ*umuD E. coli* cells. CSM and ASM mutants also did not cleave after UV exposure (data not shown). Immunoblot was probed with anti-peptide antibodies against the N-terminus of UmuDAb. (B) UmuDAb CSM and ASM1 cannot complement each other for intermolecular cleavage when co-expressed in Δ*umuD E. coli* cells. Control cell lysates collected from cells expressing UmuDAb from pACYC184 (WT from pACYC2, lanes 2, 3; CSM A83Y from pACYC2AtoY, lanes 4, 5; or ASM1 S119A from pACYCStoA, lanes 6, 7) showed cleavage and a ~14 kD UmuDAb′ cleavage product for the WT but not the ASM or CSM1. Attempted complementation from

two different mutant UmuDAb forms (CSM and ASM1) co-expressed in Δ*umuD E. coli* from pACYC184 and pIX3, respectively (lanes 8 and 9) or from pIX3.0 and. pACYC184 (lanes 10 and 11), was unsuccessful in allowing cleavage (lanes 8–11). Co-expression of WT from pACYC2 with either the ASM1 or the CSM from pIX3.0, yielded a UmuDAb′ cleavage product after MMC treatment (lanes 12–15). The asterisk indicates a ~50 kD cross-reacting protein for estimating protein loading across lanes. The immunoblot was probed with polyclonal antibodies against UmuDAb. (C) Similar to panel B, UmuDAb CSM (expressed from pACYC2AtoY) and another ASM, ASM2 (expressed from pIX2KtoA) cannot complement each other for intermolecular cleavage when co-expressed in Δ*umuD E. coli* cells (lanes 7, 8). Co-expression of WT from pACYC2 with either ASM2 (lanes 3, 4) or CSM (lanes 5, 6) yielded a UmuDAb′ cleavage product after MMC treatment. The immunoblot was probed with polyclonal antibodies against UmuDAb.

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Table 1

Strain and Plasmid Descriptions

E. coli Strain or Plasmid	Description	Reference/Source
Strain		
AB1157	Wild type *E. coli*, K-12	(Dewitt & Adelberg, 1962) Penny Beuning, Northeastern University
315	AB1157 Δ*umuD772::kan*; KanR. Constructed by transduction of Δ*umuD772::kan* from *E. coli* JW1172 (Keio collection; Baba *et al.*, 2006) into AB1157	Penny Beuning, Northeastern University
AB2463	AB1157 *recA13*	(Howard-Flanders & Theriot, 1966) Leslie Gregg-Jolly, Grinnell College
DH5α	*recA1 fhuA2* Δ*(argF-lacZ)U169 phoA glnV44* *Φ80* Δ*(lacZ)M15 gyrA96 relA1 endA1 thi-1 hsdR17*	Invitrogen
Plasmid		
pJH1	2.2 kbp of *A. baylyi* strain ADP1 chromosomal DNA containing the *ddrR* – *umuDAbC′* region cloned into pUC19; AmpR[a]	(Hare *et al.*, 2006)
pIX2	*umuDAb* 612 bp ORF cloned on *Xba*I and *Bam*HI ends into the Qiagen EasyExpress pIX3.0 vector; AmpR[a]	This study
pIX2AtoY	pIX2 bearing site directed mutation of *umuDAb* codon 83 (GCT) to TAT, yielding A83Y mutation of UmuDAb; AmpR[a]	This study
pIX2GtoE	pIX2 bearing site directed mutation of *umuDAb* codon 84 (GGT) to GAC, yielding G84E mutation of UmuDAb; AmpR[a]	This study
pIX2StoA	pIX2 bearing site directed mutation of *umuDAb* codon 119 (TCT) to GCT, yielding S119A mutation of UmuDAb; AmpR[a]	This study
pIX2KtoA	pIX2 bearing site directed mutation of *umuDAb* codon 156 (AAA) to GCC, yielding K156A mutation of UmuDAb; AmpR[a]	This study
pACYC2	*Xba*I - *Bam*HI *umuDAb* insert of pIX2, cloned into *Xba*I and *Bam*HI sites of pACYC184; CamR[a]	This study
pACYCAtoY	*Xba*I - *Bam*HI *umuDAb* insert of pIX2AtoY, cloned into *Xba*I and *Bam*HI sites of pACYC184; CamR[a]	This study
pACYCStoA	*Xba*I - *Bam*HI *umuDAb* insert of pIX2StoA, cloned into *Xba*I and *Bam*HI sites of pACYC184; CamR[a]	This study
pACYCKtoA	*Xba*I - *Bam*HI *umuDAb* insert of pIX2KtoA, cloned into *Xba*I and *Bam*HI sites of pACYC184; CamR[a]	This study

[a]KanR = resistant to kanamycin, used at 25 μg/mL; AmpR = resistant to ampicillin, used at 100 μg/mL; CamR = resistant to chloramphenicol, used at 35 μg/mL