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Characterization of cell death in *Escherichia coli* mediated by XseA, a large subunit of exonuclease VII[§]

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Exonuclease VII (ExoVII) of *Escherichia coli* is a single strand-specific DNA nuclease composed of two different subunits: the large subunit, XseA, and the small subunit, XseB. In this study, we found that multicopy single-stranded DNAs (msDNAs), Ec83 and Ec78, are the *in vivo* substrates of ExoVII; the enzyme cuts the phosphodiester bond between the fourth and fifth nucleotides from the 5′ end. We used this msDNA cleavage to assess ExoVII activity *in vivo*. Both subunits were required for enzyme activity. Expression of XseA without XseB caused cell death, even though no ExoVII activity was detected. The lethality caused by XseA was rescued by surplus XseB. In XseA-induced death, cells were elongated and multinucleated, and their chromosomes were fragmented and condensed; these are the morphological hallmarks of apoptotic cell death in bacteria. A putative caspase recognition sequence (FVAD) was found in XseA, and its hypothetical caspase product with 257 amino acids was as active as the intact protein in inducing cell death. We propose that under ordinary conditions, XseA protects chromosome as a component of the ExoVII enzyme, but in some conditions, the protein causes cell death; the destruction of cell is probably carried out by the amino terminal fragment derived from the cleavage of XseA by caspase-like enzyme.

Keywords: exonuclease VII, XseA, apoptosis, msDNA, ExoVII, retron, bacterial cell death, apoptosis-like cell death

Introduction

In *Escherichia coli*, at least four nucleases are capable of degrading single-stranded DNA (ssDNA) (Viswanathan and Lovett, 1998): exonuclease I (ExoI), exonuclease VII (ExoVII), exonuclease X (ExoX), and RecJ exonuclease (RecJ). Whereas ExoX can digest both ssDNA and dsDNA, the three other nucleases are specific to ssDNA. ExoI degrades ssDNA with 3′ to 5′ polarity, RecJ does so in the opposite direction, and ExoVII degrades in both directions (Chase and Richardson, 1974a, 1974b). These ssDNA-specific nucleases have roles in DNA recombination and repair, and their functions overlap (Viswanathan and Lovett, 1998; Burdett *et al.*, 2001).

It has been proposed that ExoVII of *E. coli* is a pentamer, composed of one large subunit (XseA) and four small subunits (XseB) (Vales *et al.*, 1982). However, a recent study by Polesza *et al.* (2012) demonstrated that ExoVII could form much larger oligomers with an estimated molecular weight of about 660 kDa. From size-exclusion chromatography data, they proposed that *E. coli* ExoVII could be a heptamer, consisting of one XseA and six XseB subunits. This was also supported by their bioinformatics-based prediction (Poleszak *et al.*, 2012). These somewhat disagreeing data suggest that there can be several forms of ExoVII, with different subunit compositions in cells. In *Neisseria meningitidis*, expression of XseB, but not of XseA, is up-regulated upon interaction with host cells, indicating an alteration of the ratio XseB/XseA. This change increased the bacterium's ability to repair their DNA (Morelle *et al.*, 2005).

A retron is a bacterial genetic element that encodes reverse transcriptase (Lim and Maas, 1989; Sherratt, 1995). Reverse transcription in retrons produces msDNA (multicopy single-stranded DNA), a short, stable ssDNA with its 5′ end covalently attached to RNA via a 2′-5′ phosphodiester bond (msDNA-RNA, see Fig. 1). Recently, a retron's ability to produce ssDNA *in vivo* was exploited to construct a bacterial system for recording a cell's experiences on its chromosome (Farzadfard and Lu, 2014). In some retrons, msDNA is cleaved from the associated RNA, leaving a short hairpin-structured ssDNA as a final product (Lim, 1992; Lima and Lim, 1997). Endonucleolytic cleavage takes place between the fourth and fifth nucleotide of the msDNAs of two retrons, Ec83 and Ec78 (Ec stands for *E. coli*, and the number indicates the length of msDNA produced by each retron). Of note, base substitution mutations in the hairpin structure of Ec83 can prevent its cleavage (Kim *et al.*, 1997).

In this study, we investigated msDNA cleavage in *E. coli* and found that ExoVII was the culprit for the endonucleolytic cleavage of both the Ec83 and Ec78 msDNAs. We also discovered that XseA protein alone did not have nuclease activity, but its production effectively killed host cells. Cells undergoing XseA-mediated death exhibited characteristics of apoptotic cell death, namely, chromosome fragmentation, chromosome condensation, and inhibition of cell division. We found a possible caspase recognition sequence that was strongly conserved among *xseA* genes. Deletion clones yielding an amino-terminal fragment similar to the hypothetical caspase-cleaved peptide effectively caused cell death. We propose that under apoptotic conditions, the amino-terminal domain of XseA is released from ExoVII through proteolysis

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by a caspase-like protease and that the N-terminal fragment functions in the apoptosis-like cell death of *E. coli*.

Materials and Methods

Strain construction

All strains used in our experiments were derivatives of *E. coli* BW25113 (Baba *et al.*, 2006) or *E. coli* EPI300 (Epicentre) and are listed in Table 1. Donor strains for P1 transduction were from the Keio collection (Datsenko and Wanner, 2000). To introduce a deletion into a recipient strain, the strain in which our target gene was replaced with Km-resistance (KmR) was chosen from the Keio collection and was used as a donor for P1 transduction. To introduce a wild-type gene, the strain where the gene adjacent to the target was replaced with KmR was used as a donor. In this case, KmR and the target were co-transduced. To construct SSU301 (*recA*$^+$ of EPI300), first the wild-type *recA* was cloned into a plasmid by PCR, and EPI300 harboring this *recA*$^+$-containing plasmid was used as a recipient for P1 transduction of *recA*$^+$. To transfer the T7 polymerase of BL21(DE3), the KmR marker on *cydB* of JW0723 was transferred to BL21 (DE3), and then this BL21(DE3)-derivative marked with KmR was used as a donor. From Km-resistant transductants, strains with the T7 polymerase gene were selected.

DNA cloning

To clone *xse*A with its promoter, four primers (XseAUPfor: 5′-GCCCGGGCATTATACAGAGCGTAACCG; 2ndNheRev: 5′-gctagcTGGTTGCGTGAAATTAGAA; 2ndNheFor: 5′-gctagcGGTCACTGGTACTTTACACT; and XseARevStop: 5′-AAGCTTAATGCACCTTTTTACGCGATTT) were used to amplify the N-terminal or C-terminal end of XseA. The two amplified fragments were independently cloned into pGEM-t Easy and then joined together using the NheI site introduced into the primers. The joining was carried out in the single-copy plasmid vector pCC1fos (Epicentre). Expression vectors containing the pSC101 replicon and T7 promoter were constructed by joining PCR fragments of pKD46 (Datsenko and Wanner, 2000) and pET21a (Novagen). To construct five different domain-deleted variants, four primers (XseA1metfor: 5′-aatgacatATGTTACCTTCTCAATCCC; XseA102For: 5′-aatgacaTATGCAGCCGGCCGGTGA;

Table 1. Strains and plasmids used in this study*

Name	Relevant characteristics	Source or method
BW25113	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514	Baba *et al.* (2006)
JW1993	sbcB::Km derivative of BW25113	Datsenko and Wanner (2000)
JW0412	xseB::Km derivative of BW25113	Datsenko and Wanner (2000)
JW1833	exoX::Km derivative of BW25113	Datsenko and Wanner (2000)
JW2860	recJ::Km derivative of BW25113	Datsenko and Wanner (2000)
JW2493	xseA::Km derivative of BW25113	Datsenko and Wanner (2000)
JW0723	cydB::Km derivative of BW25113	Datsenko and Wanner (2000)
EPI300	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (StrR) nupG trfA dhfr	Epicentre
SSU300	ΔxseB derivative of EPI300	Recombination with PCR product
SSU301	recA+ derivative of EPI300	P1Tx. D, JW2664; R, EPI300
SSU303K	ΔxseB derivative of SSU301	P1Tx. D, JW0412; R, SSU301
SSU307K	ΔxseA derivative of SSU500K	P1Tx. D, JW2493; R, SSU500
SSU401K	ΔxseB derivative of SSU500K	P1Tx. D, JW0412; R, SSU500
SSU500K	BW25113(DE3)	P1Tx. D, BL21(DE3); R, BW25113
pT110	Ec78 retron	Lima and Lim (1997)
pT161	Ec83 retron	Lim (1992)
pT14	Ec86 retron	Lim and Maas (1989)
pCC1fos	A CmR plasmid with ori2 and oriV	Epicentre
pCC-XseA	pCC1fos containing xseA	This study
pCC-XseB	pCC1fos containing xseB	This study
pCC-XseAB	pCC1fos containing xseA and XseB	This study
pT-XseB	pGEM-T Easy with xseB	This study
pSU101	AmpR, pSC101 replicon	This study
pCC-XseA (1-257)	pCC1fos containing truncated xseA (1 - 257 aa)	This study
pSU300	pSU101 with xseA ORF under T7 promoter, AmpR	This study
pSU103	CmR, pSC101 replicon	This study
pSU301	pSU103 with xseA ORF under T7 promoter	This study
pT-yifK	pGEM-T Easy with yifK	This study
pCC-XseA155	pCCfos containing xseA with D155N substitution	This study
pCC-XseA188	pCCfos containing xseA with A188T substitution	This study
pCC-XseA237	pCCfos containing xseA with G237R substitution	This study

*Abbreviations used in the table: P1Tx, P1 transduction; D, donor, R, recipient

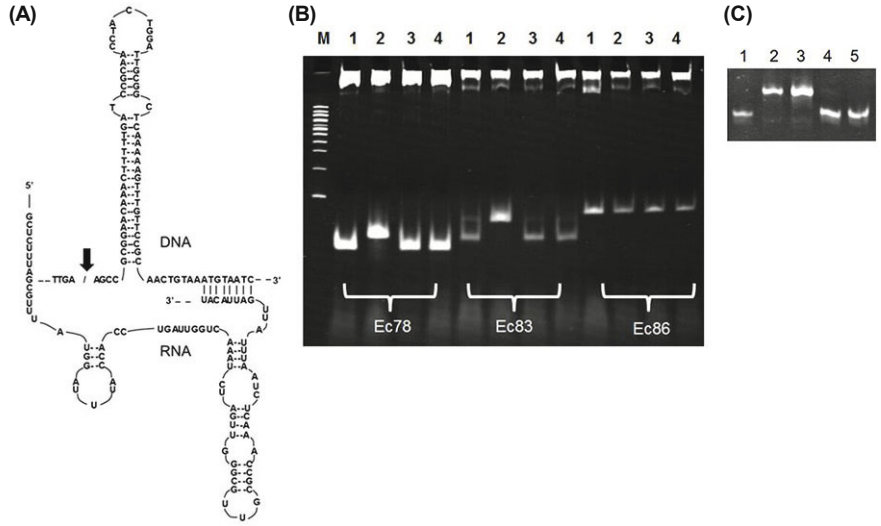

Fig. 1. MsDNA cleavage in nuclease mutants. (A) Structure of msDNA Ec83. The arrow indicates the cleavage site. Ec78 and Ec86 have similar structures. Like msDNA Ec83, msDNA Ec78 is cleaved between the fourth and fifth nucleotides from the 5′ end of the DNA, but msDNA Ec86 is not cleaved and is present as an msDNA-RNA compound. (B) Analysis of msDNA cleavage in four nuclease mutants. MsDNA was extracted from each mutant and examined on a 10% polyacrylamide gel. All strains were BW25113 derivatives with the following mutations: Lanes 1, *sbcB*; 2, *xseB*; 3, *exoX*; and 4, *recJ*. M, size marker. Note that, for both Ec78 and Ec83, the msDNA in the xseB strain was slightly larger than that in other strains. (C) Complementation of msDNA cleavage in ExoVII mutants with a plasmid containing wild type genes. msDNA was extracted from: Lanes 1, BW25113/pT161; 2, BW25113 (Δ*xseB*)/pT161; 3, BW25113 (Δ*xseA*)/pT161; 4, BW25113 (Δ*xseB*)/pCCxseB/pT161; 5, BW25113 (Δ*xseA*)/pCCXseA/pT161.

XseA267Rev: 5′-tgcataagcttaCTGATTACGGCTCACTACTT; and XseA351Rev: 5′-tgcataagcttaTTGCTGATTCAGCCGC TGT) were used. To test the activities of domain-deleted mutants, each amplified fragment was introduced into pCC1fos with its own promoter or into p15A with the pBAD promoter. For protein expression assays, intact *xseA* or the N-terminal domain with 1–257 amino acids was inserted under the control of the T7 promoter of pSU101 which had the pSC101 replicon.

Microscopy

Cultures were grown at 30°C in a fresh LB medium with shaking and divided into two when they reached an OD_{600} value of 0.4–0.5. IPTG (final concentration, 1 mM) was added to one aliquot of each culture, and incubation was continued for 2 hours. Cells were prepared and stained with DAPI as described (Zahradka *et al.*, 2009). The cells were observed with an Olympus IX 71 microscope adjusted for combined phase contrast and fluorescence microscopy. The fluorescence microscope was also equipped with a DAPI filter, FITC filter, TX-RED filter, and an Olympus DP71 microscope digital camera.

Miscellaneous methods

To amplify the copy number of pCC1fos derivatives, arabinose (final concentration, 0.02%) was added at the early logarithmic phase (OD_{600} = 0.4–0.5) (Wild and Szybalski, 2004). For lethality tests, bacterial cultures were serially diluted in LB medium, and 10 μl of each dilution was spotted on plates. MsDNA was extracted and analyzed on polyacrylamide gels as described previously (Lim and Maas, 1989; Lim, 1992).

Results

Exonuclease VII cleaves msDNA

To determine which of the four nucleases capable of degrading ssDNA was responsible for the msDNA cleavage, msDNA-producing plasmids were introduced into strains harboring a deletion of each nuclease gene. As shown in Fig. 1, Ec78 and Ec83 were cleaved efficiently in ExoI$^-$ (*sbcB*), ExoX$^-$ (*exoX*), and RecJ$^-$ (*recJ*) strains. However, neither msDNAs were cut in the ExoVII$^-$ (*xseB*) strain. No change in msDNA Ec86, a branched msDNA-RNA compound (Lim and Maas, 1989), was observed in any mutant strain. These results indicate that Ec78 and Ec83 were cleaved by ExoVII. Because ExoVII is composed of two different subunits encoded by *xseA* (large subunit) and *xseB* (small subunit), we also tested for msDNA cleavage in an *xseA* mutant. As expected, msDNA Ec83 was not cut in either the *xseA* or *xseB*

Table 2. Mutations observed in *xseA* PCR clones

Clone[a]	Observed mutation[b]	Comment
1	Large deletion (from -125 to -11)	Deletion of entire promoter
2	-36T → C	Promoter-element mutation
3	L88R (CTC → CGC), -51C → T	L88 is moderately conserved
4	G237R (GGG → AGG)	G277 is in a glycine-rich motif
5	D155N (GAT → AAT)	D155 is highly conserved
6	A188T (GCC → ACC)	A188 is highly conserved
7	W31R (UGG → CGG), H300R (CAU → CGU)	W31 is highly conserved
8	V19A (GUU → GCU), F39S (UUC → UCC), S440G (AGU → GGU)	S440 is highly conserved

[a] Each clone was isolated from independent PCR reactions.
[b] The A of the initiation codon ATG is +1

mutant strain. When a plasmid containing the wild-type copy of the deleted gene was introduced into each mutant strain, the msDNA cleavage was observed once again (Fig. 1C). Taken together, these data indicate that the cleavage of Ec78 and Ec83 is carried out by ExoVII, and this reaction requires both subunits.

XseA cannot be cloned into a multicopy plasmid

For further characterization of msDNA cleavage, we attempted to clone *xseA* and *xseB*. We could easily isolate plasmids containing *xseB* by T-vector cloning of PCR products. However, when we attempted to clone *xseA*, we were not able to obtain plasmids containing the wild-type gene; every plasmid obtained contained a mutant form of *xseA*. Table 2 displays the DNA sequence analysis of eight independently isolated PCR clones. Two clones had a mutation in the promoter region, and six clones contained amino acid changes in evolutionarily conserved residues. For example, the Gly to Arg mutation of clone #4 was at the most highly conserved glycine-rich motif (Larrea *et al.*, 2008; Poleszak *et al.*, 2012). The Asp at position 155, where a D to N substitution was observed in clone #5, was shown to be essential for nuclease activity (Poleszak *et al.*, 2012). From these observations, we conclude that wild-type clones are negatively selected for during the cloning process, and *xseA* in a high copy number plasmid would be toxic to host cells. This was of interest because the cloning of *xseA* is predicted to amplify only a single subunit of ExoVII. Moreover, cells harboring a plasmid encoding *xseA* have much lower ExoVII activity than wild-type cells (Chase *et al.*, 1986). To clarify this unexpected observation, we decided to clone the gene in a low copy number plasmid. To this end, the amino- and carboxy-terminal ends of *xseA* were separately amplified, cloned independently into a T-vector, and then joined together in pCC1fos, a vector with a very low copy number (Wild *et al.*, 2002). From this endeavor, we isolated an *xseA* gene with the correct DNA sequence. The clone pCC-XseA was used in a complementation test of the chromosomal deletion, and we confirmed that it restored msDNA cleavage (Fig. 1C).

Overexpression of the large subunit is lethal

The plasmid vector pCC1fos used for *xseA* cloning contained two replication origins (ori2 of F-plasmid and oriV of plasmid RK2). In a medium without an inducer, ori2 is functional, and the plasmid is present in a few copies per cell. The addition of arabinose activates oriV, and the plasmid copy number increases up to ~100 (Wild *et al.*, 2002). We took advantage of this unique property of pCC1fos vector to test the toxicity exerted by overproduced XseA protein. The plasmid pCC-XseA containing *xseA* with its own promoter was introduced into *E. coli* EPI300 ($xseA^+$ and $xseB^+$ strain), and the culture growth and cell viability were examined after plasmid amplification. Two hours after induction, the density of the culture dropped to nearly 100 colony forming units (CFU) (data not shown, but see below). To verify that the toxicity was due to the large subunit alone, *xseB* on the chromosome was deleted to yield *E. coli* SSU300. The plasmid pCC-XseA was introduced into this $xseB^-$ strain, and its growth and viability were examined. Figure 2 displays the optical density and density of viable cells (expressed as CFU/ml) of the culture. The optical density of *E. coli* SSU-300 with pCC-XseA increased continuously for about 3 h after induction, although the growth rate was lower than that of the control strain, which had only the vector. After 3 h of induction, the cell density reached its maximum and remained flat, whereas further growth was observed in the control culture. When the cell viability was measured, however, the density had dramatically dropped to about 1% of the initial value within 2 h of induction and stayed at this level for a few h. Because the colony count was carried out on plates containing chloramphenicol, the lethality might be due to the damage to the chromosome or to the plasmid. To resolve this ambiguity, we measured CFU with and without antibiotic selection (Fig. 2C). The colony numbers

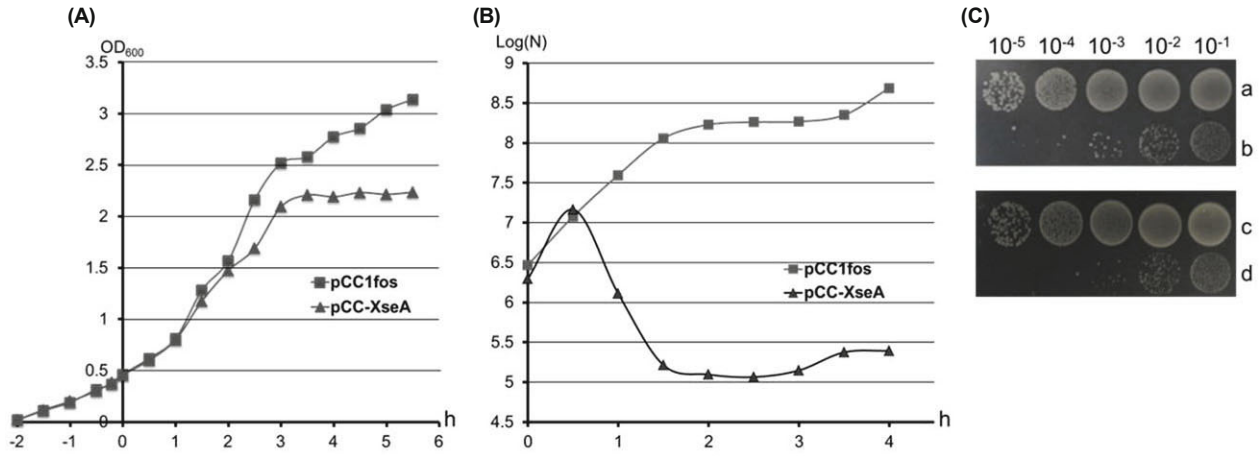

Fig. 2. Growth curve and viability of SSU300 (pCC-XseA) and SSU300 (pCC1fos). Cells were cultivated in LB with chloramphenicol, and arabinose (0.02%) was added at the early logarithmic phase to amplify plasmid copy number. (A) Growth curve, measured as OD_{600}. (B) Colony counts on LB agar with chloramphenicol. (C) Spotting of SSU300 (pCC-XseA) with (top) and without (bottom) chloramphenicol selection. Samples were taken from cultures with (b and d) and without (a and c) a 2 h induction. The numbers in X-axis of (A) and (B) indicated the hours before (negative numbers) or after (positive numbers) induction.

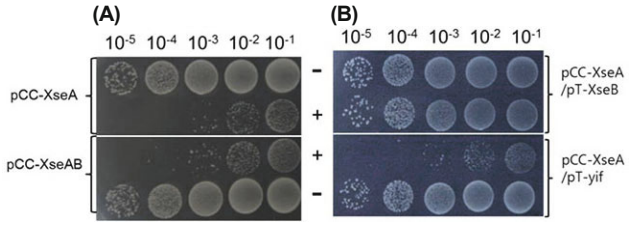

Fig. 3. Effect of XseB on the lethality of XseA overexpression. (A) Spotting of SSU300 (pCC-XseA) or SSU300 (pCC-XseAB) on plates with (+) and without (-) 2 h arabinose induction. (B) Spotting of SSU300 harboring pCC-XseA and pT-XseB (top), or of SSU300 with pCC-XseA and pT-yif (bottom), on plates with (+) and without (-) 2 h arabinose induction. The control plasmid pT-yifK was identical to pT-XseB, but contained the *yifK* gene instead of *xseB*.

obtained from the two conditions were not significantly different, with slightly more colonies on the plate without chloramphenicol. This indicated that the observed lethality was mainly due to the damage to host cells.

The small subunit counteracts the toxicity of the large subunit

The lethal effect caused by the overproduction of XseA made us question the role of the small subunit, XseB, which is believed to regulate the nuclease activity of ExoVII (Chase *et al.*, 1986; Morelle *et al.*, 2005). We constructed two plasmids: first, a DNA fragment containing *xseB* with its promoter was inserted into the plasmid pCC-XseA, resulting in pCC-XseAB containing genes of both subunits; second, the same fragment was cloned into a high copy number plasmid, pGEM-t-easy (pT-XseB; 300–400 copies/cell). In cells containing pCC-XseAB, the copy number of both genes should be the same regardless of plasmid amplification. In cells containing pCC-XseA and pT-XseB, the copy number of *xseB* would be greater than that of *xseA,* even after induction. With these constructs, we could compare the cellular effects caused by overproduction of the large subunit (pCC-XseA), both subunits in the wild-type ratio (pCC-XseAB), and overproduction of the small subunit (pCC-XseA and pT-XseB). Overproduction of both subunits with the natural gene ratio still induced host cell death (Fig. 3A). Of note, more colonies were seen in the samples with pCC-XseAB than in those with pCC-XseA, and this observation was reproducible. By contrast, a dramatic effect was observed when the gene copy number of the small subunit was greater than that of the large subunit. In samples containing both pCC-XseA and pT-XseB, overproduction of XseA by induction had little effect on the viability; CFU were not significantly different between induced cultures and non-induced cultures (Fig. 3B). This demonstrated that the biochemical activity of XseA causing cell death is counteracted by the extra amount of the small subunit, XseB.

RecA attenuates XseA-induced toxicity

E. coli SSU300, the strain used for the above experiments, is *recA*$^-$, which can cause "reckless degradation" (Zahradka *et al.*, 2009; Repar *et al.*, 2013). We reasoned that the lack of functional RecA might play a role in the observed toxicity. To address this, *E. coli* SSU303K, a *recA*$^+$ derivative of SSU300, was constructed by P1 transduction, and the toxicity of XseA in this strain was examined. As shown in Fig. 4, the CFU of cultures with the *recA*$^+$ background was still lower after induction (compared to those without induction), but the difference was small. This is in sharp contrast to the result of SSU300, in which about 99% of cells died after 2 h of induction. Thus, RecA somehow protected cells from death by XseA overexpression.

Nevertheless, even with a *recA*$^+$ host, we still encountered great difficulty in cloning *xseA* into an expression vector, such as pET21a. Therefore, we constructed a low copy number expression plasmid, pSU300, which contained the pSC101 replicon and *xseA* under the control of the T7 promoter. We found that transformation of this plasmid into SSU401K, an *xseB*$^-$ and T7 pol$^+$ strain, did not produce a colony. For successful transformation, the host strain had to be *xseB*$^+$ (or already containing a plasmid producing msDNA such as pT161, see Discussion), suggesting that the XseA from the leaky T7 promoter was still toxic and should be counteracted by XseB. This observation corroborated our previous conclusion that XseB abated XseA toxicity.

The plasmid pSU300 was introduced into SSU500K, a *recA*$^+$ *xseB*$^+$ T7 pol$^+$ strain, and the toxicity was tested. As shown in Fig. 4, cells died quickly after induction (Fig. 4B). It is worth mentioning that the XseA band was hardly discernible on an SDS-PAGE of total protein from induced cells. The onset of lethality was delayed in cells with pLysS (Fig. 4C). Thus, RecA seemed to protect cells only at low levels of XseA.

Relationship between single-stranded DNA specific nuclease and lethality

We wanted to know how msDNA cleavage and lethality changed as the ratio of large to small subunits was altered. First, we asked whether the enzymatic activity of msDNA cleavage changed with the relative increase of XseB. To answer this, plasmids pCC-xseB and pT161 were introduced into SSU303K (*xseA*$^+$ *xseB*$^-$), and msDNA was examined after the addition of arabinose. As shown in Fig. 5A, the

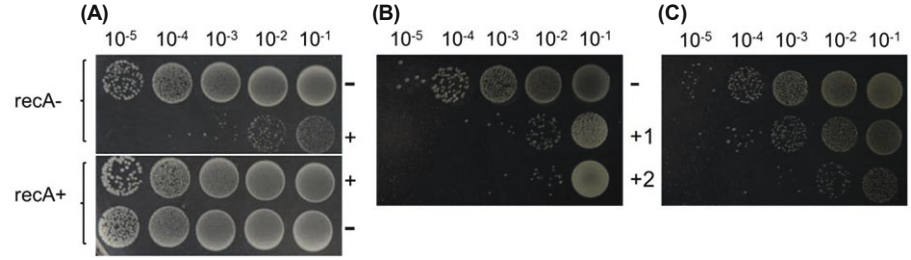

Fig. 4. Effect of RecA on the lethality caused by XseA overexpression. (A) Spotting of SSU300 (*recA*$^-$) containing pCC-XseA (top), or of SSU303K (*recA*$^+$) containing pCC-XseA (bottom), with (+) and without (-) arabinose induction. (B) Spotting of SSU500K (DE3) (*recA*$^+$, *xseA*$^+$, and *xseB*$^+$) harboring pSU300 with (+) and without (-) IPTG induction (+1, 1 h; +2, 2 h). (C) The same as (B), but the cells contained pLysS, as well as pSU300.

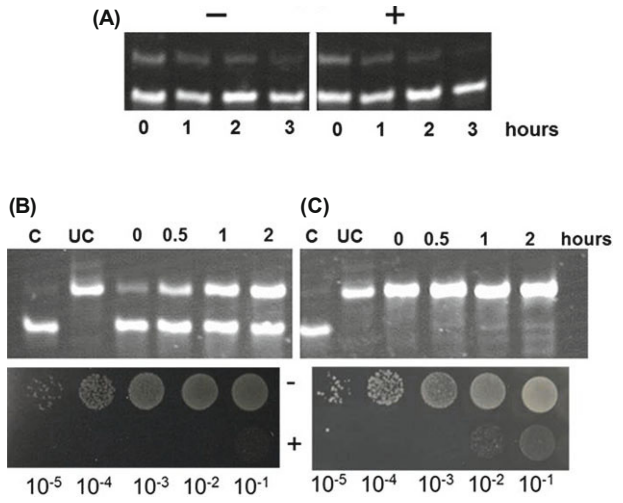

Fig. 5. MsDNA cleavage and cell death at various subunit ratios. (A) MsDNA cleavage in the presence of excess XseB. MsDNA was extracted from SSU303K (*recA*$^+$, *xseA*$^+$, and *xseB*$^-$) containing pCC-XseB and pT161, with (+) and without (-) amplification of pCC-XseB. Induction time is indicated in h. (B) MsDNA cleavage and cell death observed in SSU500K (*xseA*$^+$ and *xseB*$^+$) harboring pSU301. Lanes C and UC contain cleaved and uncleaved msDNA, respectively. (C) MsDNA cleavage and cell death observed in SSU401K (*xseA*$^+$ and *xseB*$^-$) harboring pSU301. Both SSU500K and SSU401K strains contain the T7 polymerase gene, and the plasmid pSU301 contains *xseA* under control of the T7 promoter. For msDNA analysis, samples were taken before and 0.5, 1, and 2 h after IPTG induction. MsDNA was extracted from the same OD units of cells of each culture and examined on a 10% polyacrylamide gel. To examine cell death, samples with (+) and without (-) 2 h IPTG induction were spotted after serial dilutions.

msDNA-cutting activity was not affected by the relative increase of *xseB* from the natural ratio of *xseA* and *xseB*.

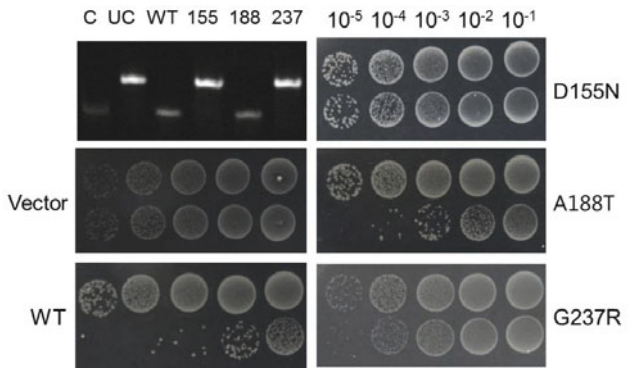

Fig. 6. MsDNA cleavage and lethality of three mutant XseA proteins. To examine msDNA cleavage by variant proteins, pCC1fos derivatives with mutant *xseA* were introduced into the *xseA* deletion strain SSU307K harboring pT161. MsDNAs were from the following: C, control msDNA from SSU500K with pT161; UC, uncut msDNA from SSU307K with pT161; and Lanes 155, 188, and 237 were from SSU307K with pT161 and a pCC1fos derivative containing a mutant *xseA* at the indicated amino acid position. For the lethality test, samples were taken from SSU300 with indicated plasmids after a 2 h induction with arabinose and spotted after serial dilutions. Panels labeled with Vector and WT were from SSU300 with pCC1fos or pCC-xseA, respectively. Panels with D155N, A188T, or G237R were from SSU300 containing a plasmid similar to pCC-XseA, but with a mutation at the indicated position.

To determine the effect of increasing levels of the large subunit, pT161 and pSU301 with *xseA* under the control of T7 promoter were introduced into either *xseB*$^+$ (Fig. 5B) or *xseB*$^-$ strains (Fig. 5C), and msDNA and cell viability were examined with and without induction. When msDNA from *xseB*$^+$ cells was examined, the majority was cleaved before induction (Fig. 5B). However, as induction proceeded and XseA accumulated in cells, the amount of the uncut form increased (upper band in Fig. 5B, top), suggesting that the total enzyme activity decreased as the large subunit increased. In the total absence of the small subunit, no msDNA digestion was observed (Fig. 5C). This indicated that the enzyme formed when there was an appropriate ratio of XseA to XseB, and in the excess of the large subunit, the activity decreased and eventually disappeared.

As expected from previous experiments, the cells died after induction regardless of chromosomal *xseB* (Fig. 5). In the absence of the small subunit, msDNA was not cleaved, even though cells died quickly (Fig. 5C). Therefore, the large subunit itself induced cell death, but both subunits in the appropriate ratio were required for msDNA cleavage.

Next, we examined msDNA cleavage and lethality of three mutants isolated during PCR cloning (D155N, A188T, and G237R; see Table 1). According to the bioinformatics study by Poleszak et al. these residues are highly conserved and located in the catalytic domain (6). As shown in Fig. 6, the D155N mutant lost the ability to cleave msDNA and displayed little lethality. In the mutant A188T, msDNA cleavage was indistinguishable from that of the wild type, and lethality was reduced; about ten times more colonies were observed from the A188T mutant than from the wild type. No msDNA cleavage was found for the G237R mutant but a low level of cell killing was still observed; the culture viability dropped to about 30% after a 2 h induction. The results

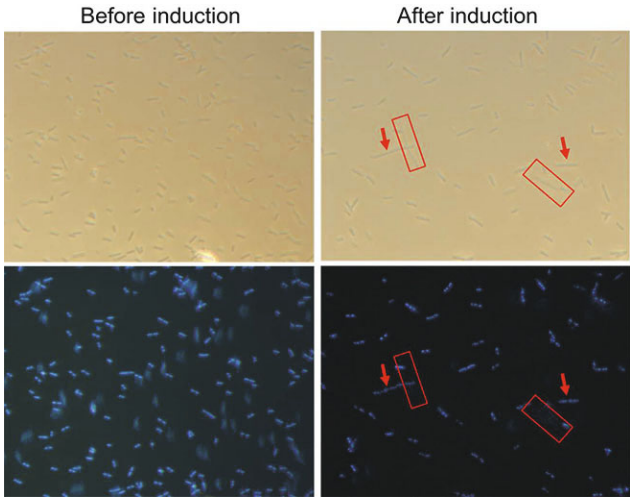

Fig. 7. Microscopic observation of cells in XseA-mediated death. *E. coli* SSU500K(DE3) with pSU300 was cultivated until early log phase, and the T7 promoter was induced by addition of IPTG. Cells were fixed in osmium tetroxide, and their DNA was stained with DAPI. They were examined by combined phase contrast and fluorescent microscopy before and after induction (2 h). Arrows indicate cells with smaller, brighter, and more localized fluorescence spots than those in healthy cells. Boxes indicate cells with no chromosomes.

showed that, although the ability to induce cell death is not identical with the single-strand DNA specific nuclease activity, the residues important for nuclease activity is also important for lethality.

Chromosome is fragmented and condensed in the cells dying by XseA

To see the characteristics of XseA-mediated cell death, we examined dying cells by microscopy. To monitor the chromosomal state, as well as cell morphology, we used fluorescence microscopy with DAPI staining. As shown in Fig. 7, many of the *xseA*-induced cells were elongated and multinucleated. Some cells lost the chromosome entirely (Fig. 7, boxed). The cells retaining the chromosome were no longer homogenously stained with the fluorescent dye. Rather, the chromosomes were fragmented and condensed, resulting in a punctate or patched staining pattern (Fig. 7, arrows). These are the morphological characteristics observed in cells undergoing apoptosis (Hakansson *et al.*, 2011; Dwyer *et al.*, 2012). Thus, *xseA*-induced cells and their nuclei had morphological characteristics commonly observed in apoptosis-like cell death.

Amino-terminal domain of XseA caused cell death

Because apoptotic effector molecules are often proteolytically activated by caspases, we considered the possibility that XseA is activated by proteolytic cleavage. We were able to locate a putative caspase recognition motif (YVAD) (Kidd, 1998) on XseA, with the sequence of 245-aD**fVAD**lrapTP-256 (capital letters indicate highly conserved residues) (Larrea *et al.*, 2008; Poleszak *et al.*, 2012). To test the hypothesis that XseA is activated by caspase cleavage, we made a mutant XseA(1–257) containing a primary structure similar to the putative caspase product and tested its lethal activity. A plasmid producing the N-terminal 257 amino acids (XseA[1–257]) did not complement chromosomal *xseA*$^-$ msDNA cleavage, but showed lethality. The killing ability of pCC-xseA(1–257) dropped the CFU as much as the wild-type plasmid did (Fig. 8B, left). The lethal effect of XseA(1–257) was not attenuated by the presence of surplus XseB (Fig. 8B, right), which was in stark contrast to the case of intact XseA (Fig. 3). This supports our hypothesis that, under ordinary conditions, XseA is a component of the ExoVII enzyme and protects cells from DNA damage, but in conditions harsh enough to cause apoptosis, the N-terminal end of XseA is cleaved off from ExoVII by a caspase-like protease, and the released XseA fragment exerts cell death, possibly by leading to chromosomal fragmentation. Four additional mutants (1–351, 107–257, 107–404, and 107–456) were also generated, and their activities were tested. None of them exhibited msDNA cleavage or lethality (Supplementary data Fig. S1).

Discussion

Bioinformatics analyses showed that *xseA* and *xseB* are widely distributed in prokaryotes, and their amino acid sequences are remarkably conserved (Larrea *et al.*, 2008; Poleszak *et al.*, 2012). Thus, these proteins are predicted to have important functions in microbial cells. ExoVII is involved in various DNA transactions, including recombination and repair, but its function overlaps with other nucleases, such as RecJ and ExoI (Harris *et al.*, 1998; Viswanathan and Lovett, 1998; Dermic, 2006). In this context, it is of interest that XseA has another function, namely, causing apoptotic-like cell death. This assertion is based on two observations; the morphological similarities of cells dying by XseA and by apoptosis, and the lethal activity of a putative caspase-product of XseA.

Bacteria can undergo apoptosis-like cell death (Carmona-Gutierrez *et al.*, 2012; Bayles, 2014). Bacterial apoptosis can be induced by various conditions (Hakansson *et al.*, 2011;

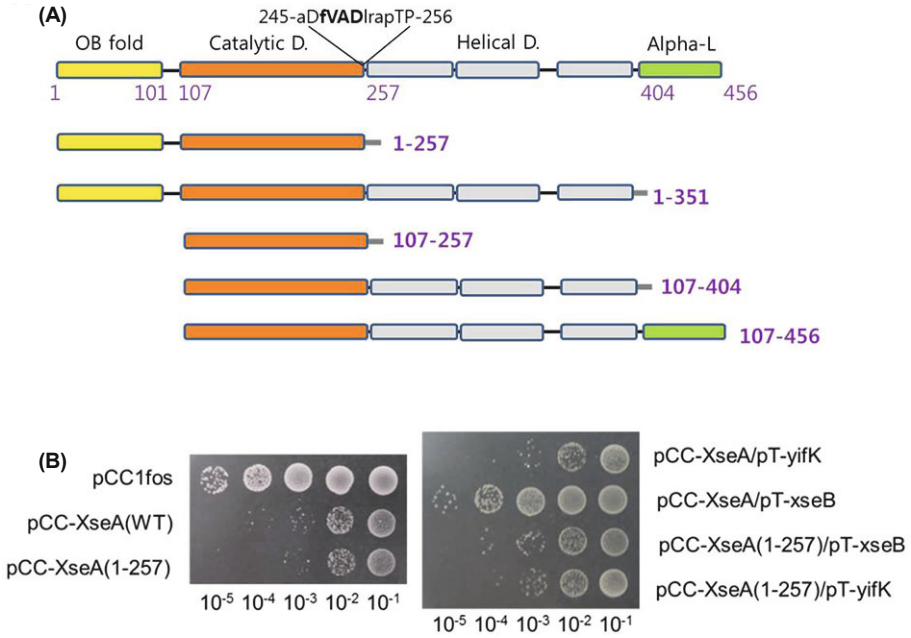

Fig. 8. Structures of deletion mutants and the lethal activity of XseA(1–257). (A) Domain structure of XseA suggested by Poleszak *et al.* (2012). The putative caspase site, 245-aD**fVAD**lrapTP-256 (capital letters indicate highly conserved residues), is indicated. (B) The lethal activity of XseA (1–257). The strain used in the experiment was *E. coli* SSU300 containing the plasmids indicated on the Fig. Cells were spotted with serial dilutions after 2 h arabinose induction. XseA(1–257) did not cleave msDNA, but killed cells. The lethal activity of XseA (1–257), unlike intact XseA, was not prevented by surplus XseB (right). Four deletion mutants (1–351, 107–257, 107–404, and 107–456) had neither msDNA cleavage activity nor lethality (Appendix Fig.).

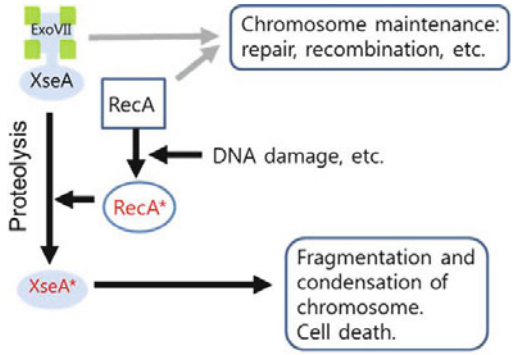

Fig. 9. Model for the function of XseA. Under ordinary conditions, together with RecA, XseA protects the chromosome as a component of the ExoVII enzyme. Under conditions harsh enough to cause apoptosis, the N-terminal end of XseA is cleaved off from ExoVII by activated RecA (RecA*), and the released XseA fragment (with 260 amino acid residues) induces cell death by fragmenting the chromosome.

Dwyer *et al.*, 2012; Erental *et al.*, 2014), but cells undergoing apoptotic death share morphological characteristics; they are often elongated and multinucleated, and their chromosomes are fragmented and condensed. Our microscopic observations of XseA-induced cells revealed such characteristics: most cells were elongated and multinucleated, and fluorescence spots were smaller, brighter, and more localized than those in healthy cells, indicating that chromosomes were fragmented and condensed. These morphological characteristics suggest that they were undergoing apoptotic cell death.

In the XseA amino acid sequence, we located a possible caspase recognition motif (YVAD)(Kidd, 1998) with the sequence of 245-aD**fVAD**lrapTP-256 (capital letters indicate conserved residues) (Larrea *et al.*, 2008; Poleszak *et al.*, 2012). Based on the analysis of residues that are conserved in the XseA family, Poleszak *et al.* (2012) made eight amino acid substitution mutants at conserved residues. Four of the mutants had no effect on ExoVII activity, suggesting that they play an important role not associated with the nuclease. We found that three of these four residues were in the hypothetical caspase recognition region (the underlined D246A, D250A, and T255A), and we believe that these residues function in the proteolytic activation of XseA in apoptotic-like cell death.

Is there a caspase-like enzyme in *E. coli*? Wadhawan *et al.* (2013) detected caspase 3 activity from *E. coli* K12 extract, and this activity increased significantly upon radiation exposure. Dwyer *et al.* (2012) screened *E. coli* proteins with the ability to bind synthetic caspase substrate peptides. They found that RecA bound the substrate analog, and the binding occurred only when RecA was activated (Dwyer *et al.*, 2012). In apoptotic-like cell death, activation of RecA is a key step (Dwyer *et al.*, 2012; Bayles, 2014), but the caspase-like activity of RecA has not been revealed nor its substrate has been identified.

Considering these reports and our observations, we propose a model for the function of XseA. Under ordinary conditions, ExoVII and RecA cooperate to protect the bacterial chromosome from DNA damage (Fig. 9). In conditions harsh enough to induce apoptotic cell death, RecA is activated and cleaves off of the N-terminal portion of XseA from the ExoVII enzyme. The released N-terminal XseA (with 260 amino acid residues) functions as a death effector, leading to chromosomal fragmentation and condensation. The fact that amino acid residues important for ssDNA-specific nuclease are also important for lethality suggests that the activated XseA may damage chromosomal DNA. This notion is also supported by the fact that RecA attenuates the lethality of XseA. Future work can test the hypothesis that activated RecA cleaves XseA at the suggested sequence, and that the cleaved product induces apoptotic cell death by acting on chromosomal DNA.

Acknowledgements

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