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Specific regions of the SulA protein recognized and degraded by the ATP-dependent ClpYQ (HslUV) protease in *Escherichia coli*

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ABSTRACT

In *Escherichia coli*, ClpYQ (HslUV) is a two-component ATP-dependent protease, in which ClpQ is the peptidase subunit and ClpY is the ATPase and unfoldase. ClpY functions to recognize protein substrates, and denature and translocate the unfolded polypeptides into the proteolytic site of ClpQ for degradation. However, it is not clear how the natural substrates are recognized by the ClpYQ protease and the mechanism by which the substrates are selected, unfolded and translocated by ClpY into the interior site of ClpQ hexamers. Both Lon and ClpYQ proteases can degrade SulA, a cell division inhibitor, in bacterial cells. In this study, using yeast two-hybrid and *in vivo* degradation analyses, we first demonstrated that the C-terminal internal hydrophobic region (139^{th} ~ 149^{th} aa) of SulA is necessary for binding and degradation by ClpYQ. A conserved region, GFIMRP, between 142^{th} and 147^{th} residues of SulA, were identified among various Gram-negative bacteria. By using MBP-SulA(F143Y) (phenylalanine substituted with tyrosine) as a substrate, our results showed that this conserved residue of SulA is necessary for recognition and degradation by ClpYQ. Supporting these data, MBP-SulA(F143Y), MBP-SulA(F143N) (phenylalanine substituted with asparagine) led to a longer half-life with ClpYQ protease *in vivo*. In contrast, MBP-SulA(F143D) and MBP-SulA(F143S) both have shorter half-lives. Therefore, in the *E. coli* ClpYQ protease complex, ClpY recognizes the C-terminal region of SulA, and F143 of SulA plays an important role for the recognition and degradation by ClpYQ protease.

1. Introduction

In *Escherichia coli*, four important ATP-dependent proteases were discovered, including Lon, FtsH, ClpAP/XP and ClpYQ (HslUV) (Gottesman, 2003). The Clp (caseinolytic protease) ATPase is a member of the AAA^+ (ATPases associated with diverse cellular activities) superfamily. Among these, ClpYQ is one of the ATP-dependent proteases (Missiakas et al., 1996). The $clpQ^+Y^+$ heat shock operon encodes a small subunit peptidase ClpQ (19 kDa) and a large subunit ClpY (49 kDa) (Chuang et al., 1993; Lien et al., 2009a).

The ClpQ core interacts only with ClpY while for other Clp proteases, the ClpP core interacts with different unfoldases, *e.g.*, ClpA, ClpX, ClpC or ClpE (Maurizi, 2008; Kress et al., 2009). A dumbbell-shaped complex ($Y^6Q^6Q^6Y^6$), which constitutes two ClpQ and two ClpY hexamers, was revealed using electron microscopy as well as the X-ray crystal structure and biochemical analyses (Kessel et al., 1996; Rohrwild et al., 1997; Bochtler et al., 2000; Wu et al., 2008). In the presence of ATP, ClpY delivers the substrates from outside the hexameric chamber into the catalytic core, wherein ClpQ degrades the substrates in the ClpYQ complex (Kessel et al., 1996; Missiakas et al., 1996; Yoo et al., 1996; Rohrwild et al., 1997; Lien et al., 2009b).

Crystal structure analyses indicate that ClpY consists of three domains, N, I and C domains (1E94, Protein Data Bank) (Bochtler et al., 2000; Song et al., 2000). The N domain is an ATPase, I domain is protruding out for gripping of the substrates (Wang et al., 2001a, Wang et al., 2001bb; Azim et al., 2005; Lien et al., 2009b) and the C domain is responsible for ClpY self-oligomerization and activation of ClpQ (Ramachandran et al., 2002; Seong et al., 2002; Lee et al., 2003; Lien et al., 2009b). Several proteins have been identified as the substrates for the ClpYQ protease, such as SulA, RcsA, RpoH, TraJ, RNaseR and YbaB (Khattar, 1997; Kanemori et al., 1999; Wu et al., 1999; Kuo et al., 2004; Lau-Wong et al., 2008; Liang and Deutscher, 2012; Chang et al., 2016; Tsai et al., 2017). The ClpQ dodecamer has 12 potential active sites, maximally six of which are sufficient for the degradation of the substrates (Lee et al., 2009). In addition, the asymmetric hydrolysis of three or four ATPs in ClpY hexamer are required for the binding/

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unfolding/translocation and degradation of substrates by ClpQ (Yakamavich et al., 2008). The pore I site (90 ~ 93 aa; GYVG) and the pore II site (265 ~ 269 aa; GESSG) in the N domain of ClpY are in the center of its hexameric circle (Wang et al., 2001a, Wang et al., 2001b; Zolkiewski, 2006). Both *in vivo* and *in vitro* experiments have shown that the ClpY pore I site is required for substrate translocation with ClpQ in the presence of ATP(s) (Park et al., 2005; Hsieh et al., 2011). In addition, yeast two-hybrid and surface plasmon resonance (SPR) analyses have identified the ability of ClpY to bind to its natural substrate, SulA or the MBP-SulA fusion protein (Lee et al., 2003; Azim et al., 2005). The two-molecule dynamic scale modeling has indicated that there is a unidirectional translocation of the threaded single polypeptide substrates *via* the central pore I site or the four-helix bundle protein substrates are delivered into the core site *via* the ancillary domain I (Koga et al., 2009; Kravats et al., 2011).

Based on the bioinformatic and phylogenetic analyses, ClpYQ is found in almost all eubacteria (Ruiz-Gonzalez and Marin, 2006; Wu et al., 2008). In *E. coli,* ClpYQ along with Lon and ClpAP are the three most important ATP-dependent proteases for intracellular protein degradation with extreme specificity (Maurizi, 1992; Jain and Chan, 2007). Also, ClpYQ is mainly responsible to degrade the misfolded proteins during the heat-shock response (Chuang et al., 1993; Missiakas et al., 1996; Wu et al., 2008; Lien et al., 2009a). In addition, in *Bacillus subtilis,* ClpY-ClpQ protease regulates multicellular development phenotypes, including motility, chemotaxis, production of secreted γ-poly-DL-glutamic acid and biofilm formation (Yu et al., 2018). Both Lon and ClpYQ can recognize SulA and degrade it (Kanemori et al., 1997; Khattar, 1997; Kanemori et al., 1999; Wu et al., 1999). SulA is easily aggregated and precipitated both *in vivo* and *in vitro* (Sonezaki et al., 1995; Jubete et al., 1996) and ClpY, alone has the ability to prevent its aggregation (Seong et al., 2000). The predicted tertiary structure of SulA contains four α helices and five β sheets (Fig. 1A). Interaction of SulA with FtsZ inhibits cell division, leading to filamentous shapes of bacterial cells and often lethality (Huisman and D'Ari, 1981; Huisman et al., 1984). FtsZ can also protect SulA from degradation by the ClpYQ protease (Wu et al., 1999; Seong et al., 2000). Crystal structure reveals that FtsZ binds to the SulA dimer, one molecule to each monomer (Cordell et al., 2003). An oligopeptide comprising the C-terminal eight residues of SulA (SA8) (Fig. 1B) interacts with Lon and can competitively inhibit the degradation of MBP-SulA by Lon protease (Ishii et al., 2000). Also, the C-terminal 169th histidine residue (Fig. 1B) is specifically recognized by Lon protease (Ishii and Amano, 2001). However, the sites of SulA recognized by ClpYQ and how ClpYQ interacts with SulA at the molecular level remain unclear.

Previous hydrophobicity analysis showed that SulA has highly hydrophobic amino acid residues, and the results from the *in vitro* pull-down assays indicated that the C-terminal 139th to 149th (C20 ~ 30aa) residues of SulA are necessary for interaction with ClpY (Hwang et al., 2014). In this study, we used a yeast two-hybrid system to demonstrate that the last 20 ~ 30 aa residues (139th ~ 149th) at the C-terminus of SulA are necessary for association with ClpY, and, this region also has an effect on degradation of SulA by ClpYQ. Analysis of SulA amino acid sequences from different Gram-negative bacteria revealed a conserved region between 142th and 147th residues, $G^{142}F^{143}I^{144}M^{145}R^{146}P^{147}$. Using yeast two-hybrid analysis, we found that amino acid substitution of either F143, I144 or M145 alters the binding affinity of SulA toward ClpY(M187I). Mutations at F143 and I144 also had effects on degradation of SulA by ClpYQ. Since SulA(I144 N) is inactive in the bacteria, our results suggest that the amino acid F143 plays an important role for recognition/interaction of SulA with ClpY and alteration of F143 is capable of influencing the half-life of SulA for its degradation by ClpYQ.

2. Materials and methods

2.1. Strains and plasmids

The yeast strain, all the bacterial strains and the major plasmids used in this study are listed in Table 1. The Gal4-based Matchmaker® Yeast Two Hybrid System from Clontech (Takara Bio USA, Inc., Mountain View, CA) was used to detect interactions between ClpY and SulA/SulA derivatives. P1*vir* phage was used to construct the YT10010 strain with *sulA* mutation and both HT1004 and HT1005 strains with *ftsZ* mutation. YT10010 was cured of its kanamycin resistance cassette with pCP20 (Cherepanov and Wackernagel, 1995). Plasmid pTH18kr (Hashimoto-Gotoh et al., 2000) was attained from the National BioResource Project (NBRP) (Arifuzzaman et al., 2006). Strain YT10010 (*lon, clpQY, cpsB*::*lacZ, sulA*) was used as a host for the EOP (efficiency of plating) test of pTH18kr-*sulA*$^+$ and its mutant derivatives. The EOP was defined as stated in the text of results. The plasmids pGilda-*clpY* (*M187I*), pB42 AD*sulA*$^+$, pTH18kr-*ha-sulA*$^+$, and pTH18kr-*mbp-sulA*$^+$ were constructed as described previously (Lee et al., 2003; Lien et al., 2009b; Hsieh et al., 2011; Chang et al., 2016). Plasmids pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ (Lien et al., 2009b) were used in an *in vivo* degradation test, which were under regulation by arabinose induction and glucose repression (Guzman et al., 1995). Expression of HA-SulA and its derivatives are from the plasmid pTH18kr-*ha-sulA*$^+$ and its derivatives under isopropyl-β-D-thiogalactopyranoside (IPTG) induction (Hashimoto-Gotoh et al., 2000). MBP-SulA and its derivatives were constructed by cloning the appropriate *sulA*$^+$ gene or its derivatives into the pTH18kr-*mbp* vector (Chang et al., 2016) using the *Eco*RI and *Bam*HI sites. The resulting plasmid pTH18kr-*mbp-sulA*$^+$ and its derivatives, in which each encoded an in-frame MBP-SulA or its derivative fusion protein in bacterial cells, were also under IPTG induction (Hashimoto-Gotoh et al., 2000).

2.2. Media and reagents

The synthetic dropout (DO) minimal medium (SD) for yeast growth, was purchased from Clontech. DO supplements, containing nucleotides and amino acid residues, were obtained from Clontech and prepared according to the manufacturer's instructions. Minimal media using different sugars were prepared as described by Miller (Miller, 1992). *E. coli* was grown in Luria broth (LB). The supplements were used as follows: 2% glucose (Glc), 2% galactose (Gal), and 1% raffinose (Raf), and 100 μg/ml ampicillin (Amp), 25 μg/ml kanamycin (Kan), 34 μg/ml chloramphenicol (Chl), or 80 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), as indicated.

2.3. Construction of sulA deletion mutants and point mutants

The DNA fragment containing open reading frame (ORF) of SulA deleted of the C-terminal 10, 20, 30 or 45 amino acids was PCR amplified using a forward primer carrying an *Eco*RI site and the start codon and a reverse primer carrying an *Bam*HI site and the stop codon at the deletion end point. These fragments were then used to construct deletion mutants *sulA*ΔC10aa, *sulA*ΔC20aa, *sulA*ΔC30aa and *sulA*ΔC45aa in pGilda. A two-step PCR was used to construct *sulA*ΔC(20 ~ 45)aa, *sulA*ΔC(20 ~ 30)aa *sulA*(F143Y), *sulA*(F143 A), *sulA*(I144 N), *sulA*(M145I) *and sulA*(R146 L). *Eco*RI-*Bam*HI DNA fragments containing the mutated genes were each constructed using two sets of primers. For example, to construct *sulA*ΔC(20 ~ 45)aa, the first-step primers F1 (5′-CGGAATTCATGTACACTTCAG

GCTATGCACATC-3′) and R1 (5′-AGAGGATGCAGTCAAATCATCTG CCAA

CCAACCGAT-3′) were used to amplify a left half fragment containing the deletion mutation of 25 bps; similarly, the primers F2 (5′ GGTTGGTTGGCAGATGATTTGACTGCATCCTCTCACGCCACGAGACAA CTTTCC-3′) and R2 (5′-GGGGGATCCTTAATGATACAAATTAGAGTG-3′)

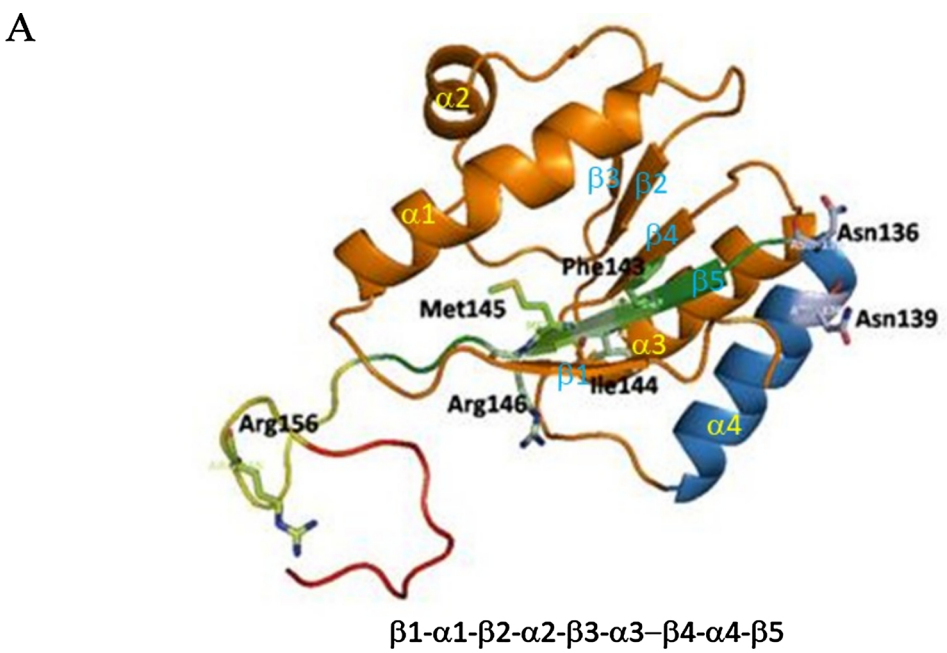

Fig. 1. The schematic diagram of *E. coli* SulA and the alignment of SulA C-terminal residues from different bacteria. (A) Using the PyMol, the configuration of SulA molecule was made by using the *Pseudomonas aeruginosa* SulA protein as the structural template (PDB code: 10FU) (Cordell et al., 2003). The red, yellow, green, and blue colors represent the last C-terminal 10 aa(s), 10–20 aa(s), 20–30 aa(s), and 30–45 aa(s). The amino acid residues are labelled as indicated in the panel. The juxtaposition of four α-helix structure and five β-sheet structure are as indicated. (B) The conserved region of amino acid sequences from the C-terminal region of SulA proteins were aligned among representative bacterial species in the *Enterobacteriaceae* family, including *Escherichia coli*, *Salmonella enterica subsp.*, *Shigella flexneri*, *Klebsiella pneumonia, Serratia proteamaculans, Yersinia pestis, Erwinia tasmaniensis* and *Citrobacter koseri.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

were used to yield a right-half fragment that overlapped the mutagenized deletion site.

The restriction sites were underlined. The resulting two overlapping fragments were then used as templates, with F1 and R2 as primers, for synthesis of the last product (the DNA fragment with 25 bps deletion), which was put in between the *Eco*RI and *Bam*HI sites of pGilda. Similarly, to construct point mutations, eg., *sulA*(F143Y), the first-step primers F1 (5′-CGGAATTCATGTACACTTCAGGCTATGCACATC-3′) and R3 (5′- GGACGCATAAT**ATA**CCCCATAGCG TTACCTTCATTT -3′) were used to yield a left-half fragment containing the mutagenized site (bold); similarly, the primers F3 (5′-TATGGGG**TAT**ATTATGCGTCCGG TAAGCGCATC-3′) and R2 (5′-GGGGGATCCTTAATGATACAAATTAGA GTG-3′) were used to yield a right-half fragment that overlapped the mutagenized site (bold) (restriction enzyme sequences are underlined). The resulting overlapping fragments were then used as templates, with F1 and R2 as primers, for synthesis of the final product, which was inserted between the *Eco*RI and *Bam*HI sites of pGilda. Other *sulA* mutants were also constructed by using the procedure described above. The newly constructed plasmids were all sequenced to confirm their correct construction. Afterwards, the DNA fragments of the directly above genes from the resultant pGilda derivative plasmids were each respectively transferred into pB42 AD at *Eco*RI-*Xho*I sites. The resultant pB42 AD derivative plasmids have each gene in-frame with the gene encoding B42 polypeptide which is an artificial transcriptional activation domain of Gal4. The plasmid pTH18kr carrying *ha -sulA*ΔC10aa, *ha-sulA*ΔC20aa, *ha-sulA*ΔC30aa, *ha-sulA*ΔC45aa, *sulA*ΔC(20 ~ 45)aa and *sulA*ΔC(20 ~ 30)aa, separately were constructed by cutting DNA *EcoRI-BamHI* fragment from pGilda *sulA*ΔC10aa, *sulA*ΔC20aa, *sulA*ΔC30aa, *sulA*ΔC45aa, *sulA*ΔC(20 ~ 45)aa and *sulA*ΔC(20~30)aa into pTH18kr-*ha* plasmid. The *mbp-sulA*(F143 A), *mbp-sulA*(F143Y), *mbp-sulA*(I144 N) and *mbp-sulA*(M145I) were constructed by cutting DNA *EcoRI-BamHI* fragment from pGilda-*sulA*(F143 A), *sulA*(F143Y), *sulA*(I144 N) and *sulA*(M145I) into pMAL-c2X (Hsieh et al., 2011) and pTH18kr-*mbp* vector (Chang et al., 2016). Each one was confirmed by restriction enzyme digestion and DNA sequencing as well. pTH18kr-*mbp-sulA*(F143D), *mbp-sulA*(F143N), and *mbp-sulA*(F143S) were constructed by procedures described above by using pTH18kr-*mbp-sulA*$^{+}$ as the template and pTH18kr-*mbp* as the vector (Chang et al., 2016). The

Table 1
Yeast strains, bacterial strains and major plasmids used in the study.

Strain, plasmid, phage	Genotype	Source or reference
Yeast		
EGY48	*MATα his3 trp1 ura2 lexA$_{op(x6)}$-leu2 P8op-lacZ*	Clontech
E. coli		
XL1Blue	*recA1 endA1 lac* [F′ *proAB lacIqZΔM15* Tn*10* (Tet^r)]	Stratagene
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 recA1 relA1 endA1*	Stratagene
SG22623	*lon cpsB–lacZ* Δara *mal :: lacI^Q*	(Gottesman et al., 1985)
AC3112	*lon clpQ clpY cpsB–lacZ* Δara *mal :: lacI^Q*	(Kuo et al., 2004)
AC3114	*lon clpY cpsB–lacZ* Δara *mal :: lacI^Q*	(Kuo et al., 2004)
JW0941	BW25113 *sulA::kan*	(Baba et al., 2006)
YT10010	*sulA* in AC3112	This study
SG22224		(Wu et al., 1999)
HT1004	*ftsZ* in SG22623	This study
HT1005	*ftsZ* in AC3114	This study
Plasmids		
pGilda	P$_{GAL1}$ *HIS3 amp^r*, xpresses LexA DNA binding domain (BD)	Clontech
pGilda-*clpY*(M187I)	BD gene in frame fused with *clpY*(M187I)	(Lien et al., 2009b)
pB42AD	P$_{GAL1}$ *TRP1 amp^r*, expresses B42 polypeptide activation domain (AD), HA epitope tag	Clontech
pB42AD-*sulA$^+$*	AD-*sulA$^+$* fusion gene in pB42AD	(Lien et al., 2009b)
pB42AD-*sulA* derivatives	AD-*sulA* mutation(s) in pB42AD	This study
pBAD24	ori (pBR322) *araC* P$_{BAD}$ *amp^r*	(Guzman et al., 1995)
pBAD24-*clpY$^+$*	*clpY$^+$* in pBAD24	(Lien et al., 2009b)
pBAD33	ori (pACYC184) *araC* P$_{BAD}$ *cm^r*	(Guzman et al., 1995)
pBAD33-*clpQ$^+$*	*clpQ$^+$* in pBAD33	(Lien et al., 2009b)
pTH18kr	ori (pSC101) P$_{lac}$ *kan^r*	(Hashimoto-Gotoh et al., 2000)
pTH18kr-*ha*	*ha* tag gene in pTH18-kr	(Lien et al., 2009b)
pTH18kr-*ha-sulA$^+$*	*ha-sulA$^+$* under the P*lac* promoter	(Lien et al., 2009b)
pTH18kr-*ha-sulA* derivatives	*ha-sulA* mutation(s) under the P$_{lac}$ promoter	This study
pTH18kr-*mbp* +	*malE* gene in pTH18kr	(Chang et al., 2016)
pTH18kr-*mbp-sulA* +	*malE-sulA$^+$* fusion gene in pTH18kr	This study
pTH18kr-*mbp-sulA* derivatives	*malE-sulA* mutation(s) fusion gene in pTH18kr	This study
pMAL-c2X	*ori*(pBR322)) P$_{tac}$ *amp^r*	New England Biolab
pMAL-p-*sulA$^+$*	*malE- sulA$^+$* fusion gene in pMAL-c2X	(Higashitani et al., 1997)
pMAL-c2X-*sulA* derivatives	*malE-sulA* mutation(s) fusion gene in pMAL-c2X	This study
pCP20	*FLP$^+$* λcI857$^+$ λP$_R$ Rep^{ts} *amp^r cm^r*	(Cherepanov and Wackernagel, 1995)
phage		
P1*vir*	(Miller, 1992)	

resulting plasmids, each carrying an insert DNA fragment, were confirmed by restriction enzyme digestion and the insert DNA were sequenced.

2.4. Yeast two-hybrid analysis

Plasmid pGilda-*clpY*(M187I) with pB42 AD-*sulA$^+$* or its derivatives, were co-transformed into *S. cerevisiae* EGY48 to carry out the yeast two-hybrid assays. Interactions between the proteins expressed by the two plasmids were tested through activiation of the *lacZ$^+$* and LEU2 reporter genes in the transformed strain in the presence of galactose and raffinose. By first culturing the yeast, EGY48(p8op-*lacZ*), in a 5-mL SD/-Ura medium at 30 °C for about 14 h (the OD_{600} reached to 0.4–0.6), this yeast strain was prepared for the transformation. Firstly, yeast cells were centrifuged and washed in sterile water and 100 mM lithium acetate (LiOAc). The cell pellets were resuspended in 240 μL of 50% (v/v) polyethylene glycol (PEG). 36 μL of 1 M LiOAc and 25 μL of salmon sperm DNA, which was boiled at 100°C and placed in an ice bath for 10 min., were mixed and then added into the yeast cell suspension. At that time, 50 μL of each plasmid were added to the yeast cell mixture. The mixture was vigorously shaken for 1 min, incubated in at 30°C for 30 min, followed by heat-shock treatment at 42°C for 30 min. After heat-shock, the solution was centrifuged at 14,000 rpm for 5 s, and the supernatant was removed. The yeast cells was suspended in 100 μL of sterile water and then spreaded on growth plates containing selective growth media (SD/-Ura/-His/-Trp). After incubated for 2–3 days at 30°C, a transformed yeast colony was transferred to a new SD-Ura/-His/-Trp growth plates at 30°C for 1–2 days incubation to activate the cells. Using the methods described before (Lee et al., 2003) to measure the expression of the reporters, *S. cerevisiae* EGY48(p8op-*lacZ*) coexpressing BD and AD hybrid proteins were then subjected to the β-galactosidase assay and scored on the Leu$^+$ selective (Gal/Raf/-Ura/-His/-Trp/-Leu) plates along with the X-Gal (Gal/Raf/-Ura/-His/-Trp) plates at 30 °C for several days to observe the cell growth. Subsequently, the Western blot assays were used to detect the AD-fused protein by adopting the anti-HA monoclonal antibody using the methods as described previously (Lee et al., 2003).

2.5. Western blot assay of HA-SulA and its derivatives as well as MBP-SulA and its derivatives in the induction or no induction of the pBAD33-clpQ$^+$ and pBAD24-clpY$^+$

YT10010 (*sulA*) mutant strain carrying pBAD33-*clpQ$^+$* and pBAD24-*clpY$^+$* was transformed with pTH18kr-*ha-sulA$^+$* or its derivatives. Similarly, YT10010 (*sulA*) strain carrying pBAD33-*clpQ$^+$* and pBAD24-*clpY$^+$* was transformed with pTH18kr-*mbp-sulA$^+$* and its derivatives, separately. All the resultant transformants were selected on LA plates with chloramphenicol (17 μg/ml), ampicillin (50 μg/ml) and kanamycin (12.5 μg/ml). These transformants were used to test the degradation of an IPTG-induced SulA and its derivatives by L-arabinose induced ClpYQ protease. YT10010 (*sulA*) mutant strain carrying pTH18kr, pBAD33 and pBAD24 was used as a negative control. YT10010 (*sulA*) carrying pTH18kr-*ha-sulA$^+$* (or pTH18kr-*mbp-sulA$^+$*) or its derivatives were transformed with pBAD33 and pBAD24. The

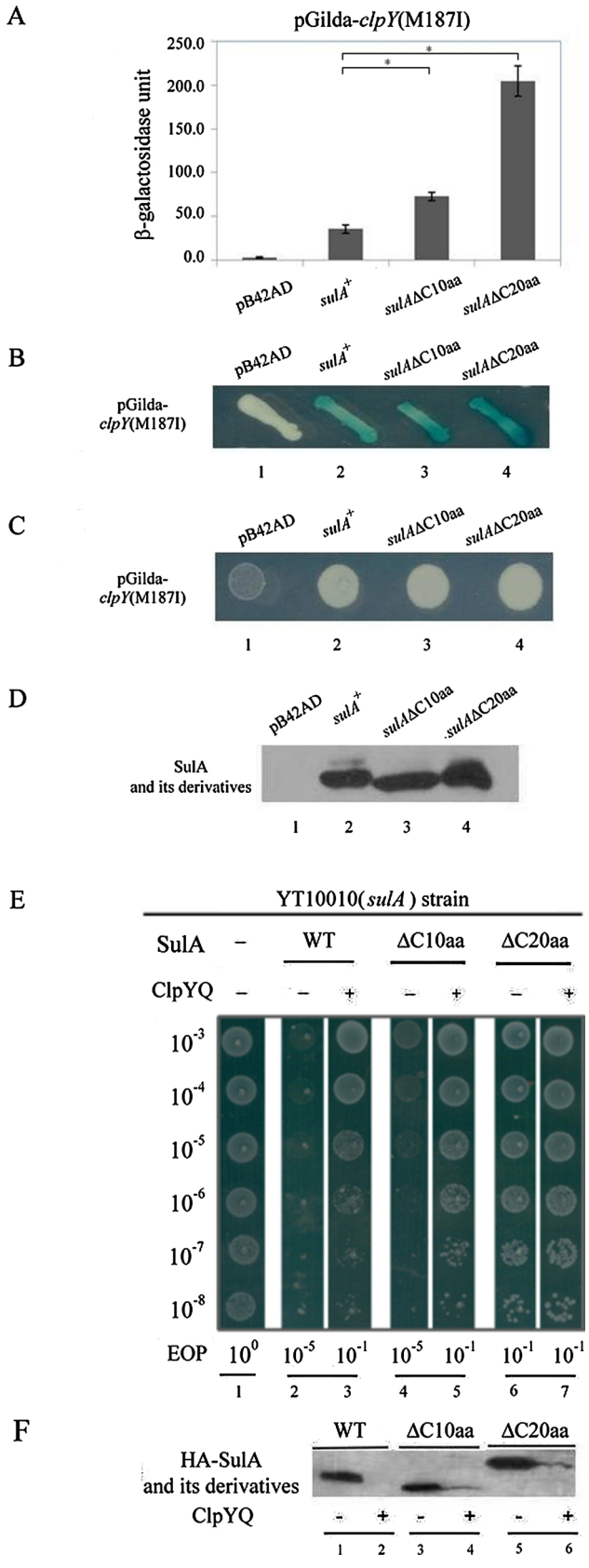

Fig. 2. Expression of *lacZ*, LEU2 in yeast EGY48(p8op-*lacZ*), each with a pair of AD and BD fusion protein. Plasmid pGilda has BD domain and pB42AD has AD domain. Each fusion protein is as indicated. As well, the degradation of SulA and its mutant derivatives by ClpYQ protease. (A) Plasmid pGilda-*clpY*(M187I) expresses BD-ClpY(M187I) fusion protein and pB42AD-*sulA*$^+$ and its derivatives express AD-SulA, AD-SulAΔC10aa, and AD-SulAΔC20aa, separately. The β-galactosidase assays were performed using yeasts carrying the above pairs of plasmids. The standard deviations were as indicated using error bars. $P < 0.05$ (the * symbol) was considered statistically significant with Student's *t*-test (B) The yeast cells were scored on the Gal + Raf-Ura-His-Trp plates containing X-Gal over 3 days. (C) The yeasts were grown on Leu$^+$ selective media. (D) The Western blot of SulA and its derivatives in the AD fusion protein was done using anti-HA antibody. (E) Each pair of the bacterial strains, which carry pTH18-*ha-sulA*$^+$, pTH18-*ha-sulA*ΔC10aa, pTH18-*ha-sulA*ΔC20aa, separately; one has pBAD33 and pBAD24 as the controls and the other has pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ for the induction of ClpYQ protease by addition of L-arabinose. The EOP value for an each control (lane 2, 4 and 6) was determined as the average number of colonies formed on the LB growth media supplemented with IPTG divided by the average number of colonies detected on the growth media without the addition of IPTG. The induction of SulA and SulAΔC10aa by IPTG in bacteria was almost lethal and led to the lower EOP (lane 2 and 4). As contrast, SulAΔC20 aa could not cause the bacterial cells lethal and led to the higher EOP (lane, 6). The EOP value for the experimental groups was determined by the average number of colonies formed on the growth media with an addition of L-arabinose on the top of an IPTG induction, divided by the colonies detected on the growth media plus L-arabinose but without an addition of IPTG (lane, 3, 5 and 7). The induction of ClpYQ protease led the cell survival and the higher EOP (lane, 3, 5 and 7). (F) Residual HA-SulA and its derivatives with or without the expression of ClpYQ protease in various bacteria. The anti-HA monoclonal antibody was used in Western blot assays.

resulting strains were each used as the positive control for the expression of its own SulA or SulA derivatives. Hence, the overnight bacterial cultures were inoculated at 1:100 dilution into LB medium with 0.5% L-arabinose and the appropriate antibiotics. After 3 h, or when OD_{600} reached 0.5, IPTG was added into the media at a final concentration of 1 mM. After another 3 h, 1 ml culture was taken and the OD_{600} was recorded. The sample loadings were normalized by OD_{600} and the equal amount of samples were electrophoresed by 12.5% SDS-PAGE. The amount of proteins loaded for each sample was determined by Coomassie Blue R-250 staining. Subsequently, Western blot analysis was equivalently performed using the monoclonal HA antibody (Roche, Indianapolis, IN, USA) or the MBP multiple antibody (NEB, Ipswich, MA, USA) to detect the HA-SulA, and MBP-SulA as well as their derivatives, respectively. Enhanced chemiluminescence (Pierce, Rockford, IL, USA) was used to detect the horseradish peroxidase activity associated with the secondary antibody during the Western blot analyses.

2.6. MBP-SulA and its derivatives in vivo degradation assay

The pTH18kr-*mbp-sulA*$^+$ and its derivatives were transformed into *E. coli* HT1004 [SG22623 with *ftsZ* mutation (Wu et al., 1999)]. The pTH18kr-*mbp-sulA*$^+$ was also transformed into *E. coli* HT1005 [AC3114 with *ftsZ* mutation [(Wu et al., 1999; Kuo et al., 2004)]. The transformed bacterial cells were overnight cultured in LB broth with an antibiotic. Next, the overnight bacterial cells were inoculated into the fresh LB media with an antibiotic and grew until the OD_{600} reached 0.5 (exponential phase) and IPTG, with 1 mM final concentration, was then added to induce protein synthesis for 30 min. Subsequently, to prevent new protein synthesis, spectinomycin (at final concentration of 150 μg/ml) was added as at time 0 and the bacterial extracts were collected at specific time intervals. The OD_{600} values of the bacterial growth media were recorded at each time point. The bacterial pellets were harvested, washed twice, and dissolved in 2X SDS-PAGE sampling buffer. Next, in Western blot analysis, the sample loadings were normalized by OD_{600} and the equal amounts of samples were electrophoresed by 12.5% SDS-PAGE. The amount of protein loadings were determined by Coomassie

Blue R-250 staining. Then, the similar loading proteins were transferred onto PVDF membrane (Thermo Scientific™, Waltham, MA, USA), MBP-SulA and its derivatives were detected by anti-MBP antibody (NEB, Ipswich, MA, USA). The Western blots were developed using Pierce enhanced chemiluminescence (ECL) system.

2.7. Statistical analysis

The β-galactosidase analyses were repeated three times, in which each sample contains three replicates. All the results were pooled together for statistical evaluation. Student's *t*-test was applied for the comparison of two interested samples. The P value < 0.05, which symbolized as an asterisk (*), was considered statistically significant in the tests.

3. Results

From a previous study, it was shown that ClpY interacted with SulA in a yeast two-hybrid assay (Lee et al., 2003). Thus, ClpY was fused in frame with the BD domain; SulA was fused in frame with AD domain, and two fused proteins associated together to activate the host reporter genes, resulting in the expression of *lacZ* and a Leu$^+$ phenotype. For that reason, yeast co-transformants carrying a pair of plasmids BD-*clpY*$^+$ and AD-*sulA*$^+$ are capable of growing on Leu$^+$-selective media and are light blue on X-Gal plates. By introducing the pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ into YT10010(*sulA*) expressing SulA or its derivative mutant proteins *via* an IPTG induction of a single copy plasmid pTH18kr-*ha* (or pTH18kr-*mbp*) *sulA*$^+$ or *sulA* variants, the degradation of SulA or SulA mutants were assayed while the bacterial co-transformant cells induced both ClpQ and ClpY expression. Thus, through yeast two-hybrid analysis and the bacterial physiological study, we tested whether ClpY associated well with SulA or SulA variants in two-hybrid assays and whether ClpY and ClpQ together are capable of degrading SulA or SulA variants in the bacteria.

3.1. ClpY(M187I) interacts with SulA and SulAΔC10aa as SulAΔC20aa in a yeast two-hybrid assay, and ClpYQ degrades all three proteins

The previous yeast two-hybrid analysis showed that the methionine to isoleucine substitution at position 187 of ClpY, ClpY(M187I), interacts with SulA strongly (Lien et al., 2009b). Thus, in this study, ClpY (M187I) was instead used to test for ClpY association with SulA or SulA deletion mutants, *i.e.* SulAΔC10aa or SulAΔC20aa. ClpY(M187I) was fused with BD domain, SulA and its mutant derivatives were fused with AD domain, separately. After co-transformation of BD-ClpY(M187I) with AD-*sulA*$^+$ (also *sulA*-deletion derivatives, separately), each resultant transformant cells were scored on X-Gal and Leu$^+$ selective media in addition to measuring β-galactosidase activity. As shown in Fig. 2, all three transformants had higher β-galactosidase activity compared to the negative control (Fig. 2A) and showed blue color on minimal media containing X-Gal (Fig. 2B) as well grew well on Leu$^+$ selective media (Fig. 2C). The Western blot assay indicated that SulA, SulAΔC10aa and SulAΔC20aa are expressed equally well in yeast cells (Fig. 2D). Therefore, ClpY(M187I) can interact with SulA, SulAΔC10aa and SulAΔC20aa. It has an enhanced interaction between ClpY(M187I) and SulAΔC20aa.

To further characterize whether ClpYQ protease can degrade SulA deletion derivatives, YT10010 (*sulA*) carrying pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ were used to test this possibility. YT10010(*sulA*) by itself grew well on the LB media (Fig. 2E, lane 1). After co-transformation with pTH18-kr-*ha-sulA*$^+$, pTH18-kr-*ha-sulA*ΔC10aa, or pTH18-kr-*ha-sulA*ΔC20aa into YT10010, the resulting bacterial cells were treated with or without IPTG and the efficiency of plating (EOP) of the each bacterial strain was determined. Mainly, the early log-phase bacterial cultures were separated into two parts. One part was treated with IPTG (1 mM final concentration) and the other part was continuously grown without addition of IPTG. After 3 h of growth, the bacterial cells were each diluted in series and plated for calculation of the EOP. The EOP value was taken as the average number of colonies formed on medium with 1 mM IPTG induction divided by the average number of colonies detected on medium without IPTG induction. As shown in Fig. 2E in the absence of ClpYQ, expression of HA-SulA and HA-SulAΔC10aa gave lower EOP (Fig. 2E, lane 2 and 4) indicating that HA-SulA and HA-SulAΔC10aa still maintain the lethal activity. The higher EOP performed by YT10010 carrying pTH18-kr-*ha-sulA*ΔC20aa implies that HA-SulA ΔC20aa lost the lethal activity (Fig. 2E, lane 6). Without an induction of ClpYQ protease, the EOP value of the above bacterial strains was separately used as the control. Next, all the bacterial strains have the higher EOP when ClpYQ was induced by L-arabinose (Fig. 2E, lane 3, 5 and 7). The subsequent Western blot analysis demonstrated that all the strains expressed SulA and its derivatives well after IPTG induction (Fig. 2F, lane 1, 3 and 5). However, HA-SulAΔC20aa had a higher molecular weight on SDS-PAGE. As results, all of the above SulA and its derivative proteins could be degraded by ClpYQ protease, (Fig. 2F, lane 2, 4 and 6).

3.2. ClpY(M187I) did not interact with SulAΔC30aa, SulAΔC45aa, and SulAΔC(20 ~ 45)aa in a yeast two hybrid assay

To further characterize SulA, determining the site responsible for association with ClpY, various C-terminal deletion mutants of SulA were constructed, *i.e.* SulAΔC30aa, SulAΔC45aa, SulAΔC(20 ~ 45)aa as well as SulAΔC(20-30)aa. Again, the genes of the above SulA mutants were fused with AD-domain gene in plasmid pB42AD, and co-transformed with BD-*clpY*(M187I) gene in plasmid pGilda into *S. cerevisiae* EGY48. After co-transformation, the resulting co-transformant cells were scored on the X-gal, Leu$^+$ selective media and assayed for β-galactosidase activity. As shown in Fig. 3A, B and C, yet again yeasts expressing ClpY(M187I)-SulA dual proteins showed the blue color on X-Gal plate, growth on Leu$^+$ selective media and had higher β-galactosidase activity. The yeasts carrying the rest of the four pair plasmids, ClpY(M187I)/SulAΔC30aa, ClpY(M187I)/SulAΔC45aa, ClpY(M187I)/SulAΔC(20 ~ 45)aa and ClpY(M187I)/SulAΔC(20-30)aa, showed white color, less growth on Leu$^+$ selective plate, and lower β-galactosidase activity (Fig. 3A, B and C). Western blot analysis showed SulA and its derivative deletion mutants were expressed well in yeasts (Fig. 3D). These results indicated that the last C-terminal 20th to 30th amino acids of SulA are required for association with ClpY. To also test whether ClpYQ protease can degrade SulAΔC30aa, SulAΔC45aa, SulAΔC(20 ~ 45)aa, as well as SulAΔC(20 ~ 30)aa, YT10010(*sulA*) carrying pBAD24-*clpY*$^+$, pBAD33-*clpQ*$^+$ was transformed with pTH18kr-*ha-sulA*$^+$, *ha-sulA*ΔC30aa, *ha-sulA*ΔC45aa, *ha-sulA*ΔC(20 ~ 45)aa, and *ha-sulA*ΔC(20 ~ 30)aa. Again, YT10010 carrying pBAD24 and pBAD33 was transformed with pTH18kr-*ha-sulA*$^+$, *ha-sulA*ΔC30aa, *ha-sulA*ΔC45aa, *ha-sulA*ΔC(20 ~ 45)aa, and *ha-sulA*ΔC(20-30)aa; separately and these transformants were used as the positive controls (Fig. 3E, lane 1, 3, 5, 7 and 9). As shown in the Western blot analysis, ClpYQ degrades SulA fully but did not completely degrade SulAΔC30aa, SulAΔC45aa, SulAΔC(20 ~ 45)aa and SulAΔC(20 − 30)aa (Fig. 3E, lane 2, 4, 6, 8 and 10).

3.3. ClpY(M187I) interacts with SulA variants (point mutants) in a yeast two-hybrid assay

Since ClpY(M187I) did not well interacted with SulAΔC(20 − 30)aa in a yeast two hybrid assay and SulAΔC(20 − 30)aa could not be fully degraded by the ClpYQ protease, it was indicated that the C-terminal 139th to 149th (C20 ~ 30aa) residues of SulA were necessary for the interaction and degradation by the ClpYQ protease. In addition, the *in vitro* binding assays also indicated that the C-terminal 139th to 149th (C20 ~ 30aa) residues of SulA were necessary for an interaction with ClpY (Hwang et al., 2014). Similarly, this region is necessary for an *in*

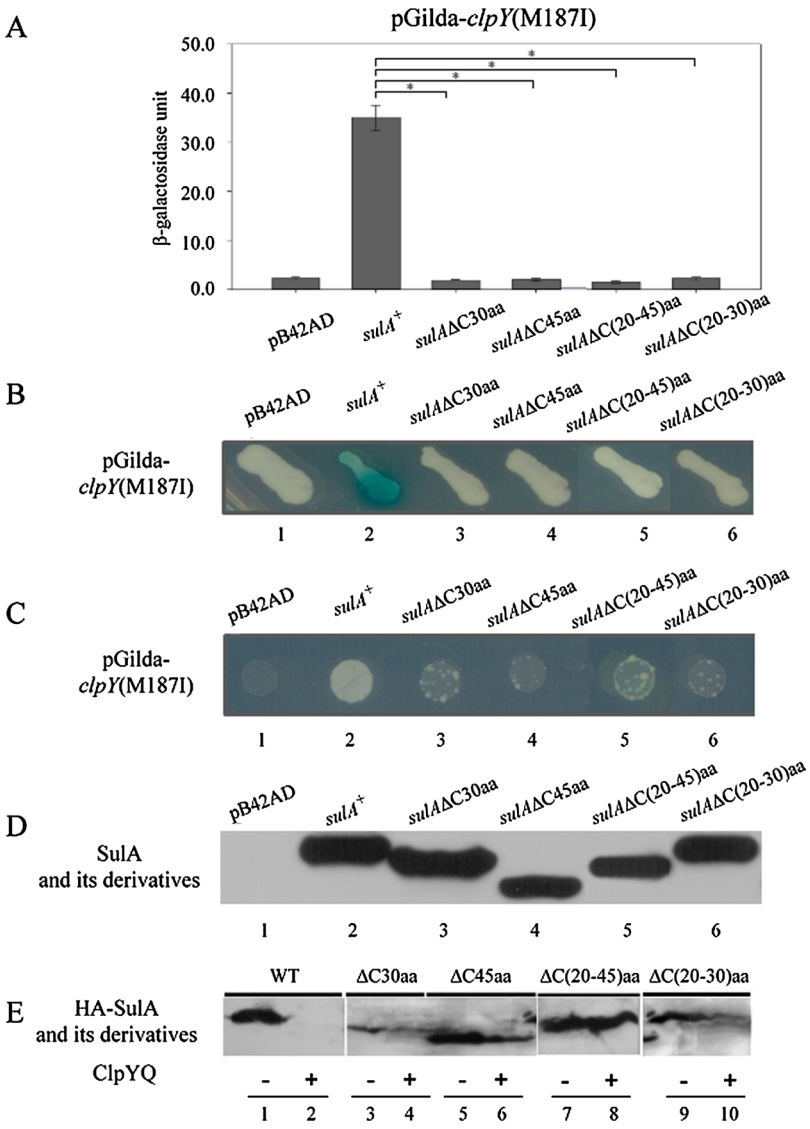

Fig. 3. Expression of *lacZ*, LEU2 in yeast EGY48(p8op-lacZ), each with a pair of AD and BD fusion proteins. Plasmid pGilda has the BD domain and pB42AD has the AD domain. Each fusion protein is as indicated. The degradation of SulA and its mutant derivatives by ClpYQ protease are shown. (A) Assays of β-galactosidase activities in yeasts. Each pair of plasmids were as indicated. The standard deviations were as indicated using error bars. $P < 0.05$ (the * symbol) was considered statistically significant with Student's *t*-test (B) Expressions of *lacZ* was scored on the Gal + Raf-Ura-His-Trp plates containing X-Gal over 3 days. (C) The yeasts were grown on Leu deficient media. (D) The Western blot of SulA and its derivatives in the AD fusion protein using anti-HA antibody. (E) Residual HA-SulA and its derivatives with or without the expression of ClpYQ protease in various bacteria. The anti-HA monoclonal antibody was used in Western blot assays.

vitro fully degradation by ClpYQ protease (Hwang et al., 2014). Besides, there is a conserved region in SulA molecule between 142th to 147th residues, $G^{142}F^{143}I^{144}M^{145}R^{146}P^{147}$ (Fig. 1B). Subsequently, we mutagenized most of the amino acid residues in this region. The rationale is to test whether an individual amino acid residue within the consensus sequences 142th to 147th residues has an effect on the recognition of SulA by ClpY. Consequently, several point mutants in this region were made in SulA, i.e. F143Y, F143A, I144 N, M145I, and R146 L. The yeast two-hybrid assays were also used to test each mutant for its affinity with ClpY(M187I). The yeast cells, which expressed ClpY(M187I) and SulA mutant, i.e., F143 A, M145I or R146 L, showed blue color on X-Gal plate and grew well on Leu$^+$ selective plate and gave higher β-galactosidase activity (Fig. 4A, B, C). However, in the presence of ClpY (M187I), with SulA(F143Y) or SulA(I144 N), yeasts had nearly white color, grew less on Leu$^+$ selective plate and lower β-galactosidase activity. Mostly, similar amount of each individual SulA point mutant molecule and SulA was detected (Fig. 4D). Therefore, most of these SulA mutants have a higher binding affinity with ClpY(M187I) except for SulA(F143Y) and SulA(I144 N), indicating that these two single point mutants have negative effect on the binding affinity toward ClpY.

3.4. The physiological effect of SulA C-terminal point mutants and the degradation of SulA C-terminal point mutants by ClpYQ protease

Subsequently, plasmid pMAL-c2X-*sulA*$^+$, pMAL-c2X- *sulA* (F143A), -*sulA* (F143Y), -*sulA*(I144 N) and -*sulA*(M145I) were transformed into YT10010(*sulA*) in series, and the EOP of each transformant was determined with or without IPTG induction. YT10010(*sulA*) grew well by itself (Fig. 5A, lane 1). As a control, the bacteria carrying pMAL-c2X-*sulA*$^+$ showed lethal effect and had lower EOP (Fig. 5A, lane 2). Similarly, the bacterial cells carrying pMAL-c2X- *sulA* (F143A), -*sulA* (F143Y) and -*sulA*(M145I) had lower EOP (Fig. 5A, lane 3, 4 and 6). Only the cell carrying pMAL-c2X-*sulA*(I144 N) has higher EOP (Fig. 5A, lane 5). All the SulA derivative mutants maintain the cellular activity except the SulA-I144 N. Also, the *in vivo* degradation of SulA point mutants were tested. YT10010 (*sulA*) carrying pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ were separately transformed with each pTH18kr-*mbp-sulA*$^+$ and pTH18kr-*mbp-sulA* derivatives, the resultant transformants were tested for the degradations of SulA and its derivatives. YT10010 (*sulA*) carrying pBAD33, pBAD24 with an individual pTH18kr-*mbp-sulA*$^+$ and its derivatives were each used as the controls. Using methods described above, SulA and its derivative mutants were expressed well (Fig. 5B, lane, 2, 4, 6, 8 and 10). Except SulA(F143Y) and SulA(I144 N), which were not fully degraded by ClpYQ protease (Fig. 5B, lane 7 and

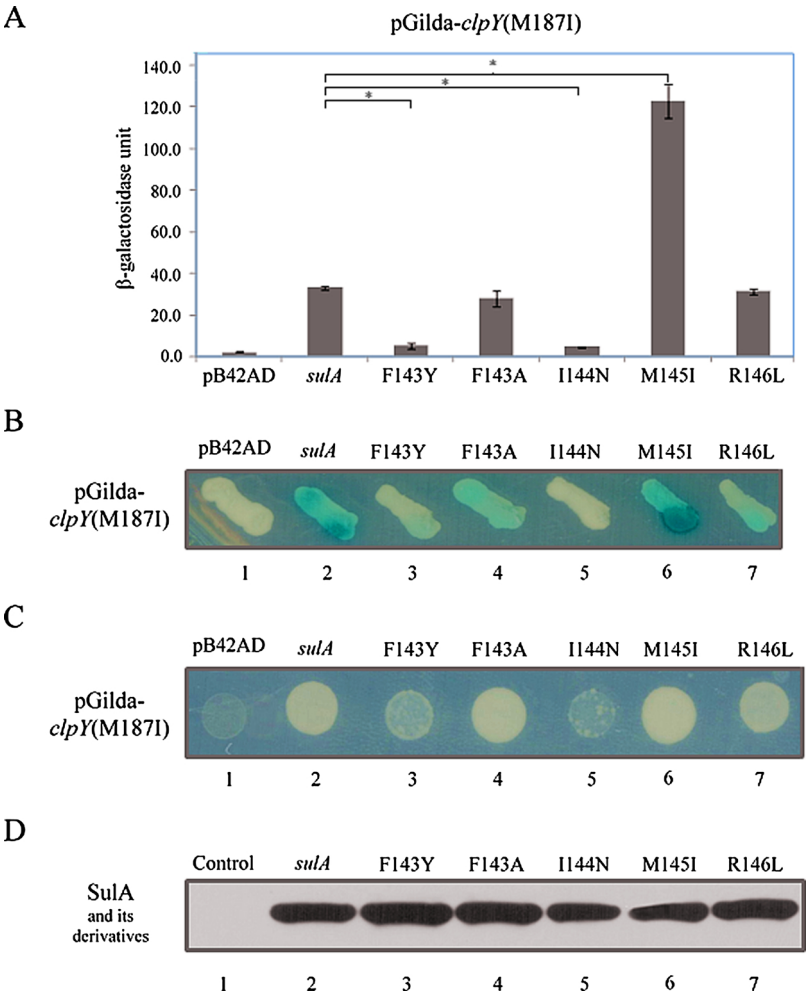

Fig. 4. Expression of *lacZ*, LEU2 in yeast EGY48(p8op-lacZ), each with a pair of AD and BD fusion proteins. Plasmid pGilda has the BD domain and pB42AD has the AD domain. (A) Assays of β-galactosidase activities in yeasts. Each pair of plasmids were as indicated. The standard deviations were as indicated using error bars. $P < 0.05$ (the * symbol) was considered statistically significant with Student's *t*-test (B) Expressions of *lacZ* was scored on the Gal + Raf-Ura-His-Trp plates containing X-Gal over 3 days. (C) The yeasts were grown on Leu deficient media. (D) The Western blot of SulA and its derivatives in the AD fusion protein using anti-HA antibody.

9), SulA and other SulA point mutants are all degraded by ClpYQ protease (Fig. 5B, lane 3, 5 and 11). Since SulA(I144N) lost its activity in bacterial cells, an abnormal structure of itself could be formed in the bacteria. So, the above results indicate that merely the 143th phenylalanine at their position of SulA is necessary for the degradation by ClpYQ protease.

3.5. Effects of various amino acids in 143th position on the degradation of MBP-SulA by ClpYQ protease

To further characterize whether F143 of SulA has an effect on the degradation of SulA by ClpYQ protease, three additional point mutants were constructed in this residue by substituting F with N, S or D and resulting pTH18kr-*mbp-sulA*(F143S), *sulA*(F143N) and *sulA*(F143D). These three plasmids and the wild-type pTH18kr-*mbp-sulA*$^+$ plasmids were transformed into a HT1004 (*lon*, *ftsZ*) mutant which has an intact *clpQ*$^+$*Y*$^+$ operon in the chromosome but lacks both Lon and FtsZ activity. Since SulA interacts with FtsZ, FtsZ could protect SulA from degradation by protease; a *ftsZ* mutation was taken on in addition to the *lon* mutation (Wu et al., 1999). Since *clpQ*$^+$*Y*$^+$ was highly expressed at 41 °C via heat shock induction (Lien et al., 2009a), the *in vivo* degradation analyses in HT1004 were performed at 41 °C. As shown, ClpYQ degrades SulA(F143D) more rapidly than it did to the wild-type SulA (Fig. 6A). ClpYQ degrades SulA(F143S) slightly faster (Fig. 6A). In contrast, ClpYQ degrades SulA(F143N) or SulAF(143Y) more slowly (Fig. 6A). These results also support that the position at 143th of SulA has an effect on the recognition/binding, translocation and degradation of the substrates by ClpYQ. In addition, we also demonstrated that ClpYQ protease is responsible for degradation of MBP-SulA or its derivatives in HT1004 (*lon ftsZ*) mutant. As observed, MBP-SulA in HT1005 (*lon clpY ftsZ*) mutant has a longer half-life than it did in HT1004 (*lon ftsZ*) mutant (Fig. 6B).

4. Discussion

Both Lon and ClpYQ, belonging to ATP-dependent proteases, are capable of degrading the SulA molecule in *E. coli*. Lon is the major protease for degradation of SulA. In contrast, ClpYQ is a redundant protease for SulA degradation (Wu et al., 1999). However, it remains unknown to distinguish the mechanisms concerning how these two proteases recognize SulA for degradation. With Lon protease, it has been shown that the C-terminal 169th histidine residue of SulA is the most important amino acid for the recognition and degradation by Lon protease (Ishii and Amano, 2001). Our yeast two-hybrid analyses also indicated that the full-length SulA molecule has a higher binding affinity toward Lon(S679A) protease, which has a mutation at position 679th in a Ser-Lys dyad proteolytic active site (Botos et al., 2004) (in S1 figure). It was shown before that Lon(S679A) could not degrade SulA

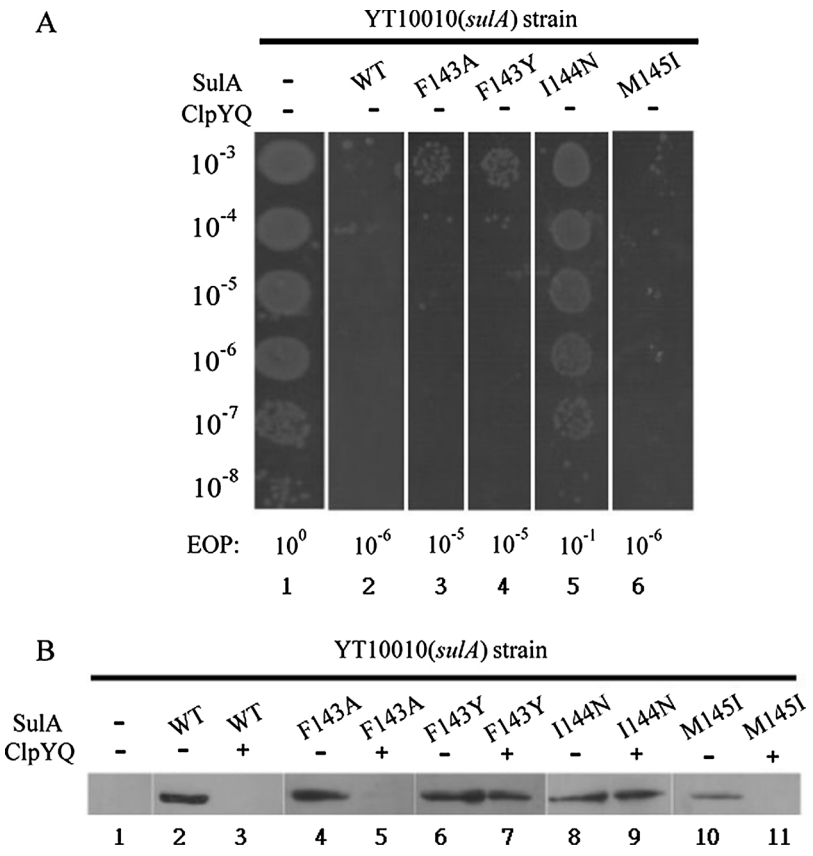

Fig. 5. The growth of the various bacterial strains, which carried the pMAL-p-*sulA*$^+$ (pMAL-c2X-*sulA*$^+$) and its single point mutation derivatives under the IPTG induction. As well, the degradation of SulA and its single point mutation derivatives by the ClpYQ psrotease. (A) Each bacterial strain, which carried the pMAL-p-*sulA*$^+$ (pMAL-c2X-*sulA*$^+$) and its derivatives, were plated on the media; separately, with IPTG induction during the growth. The EOP of each bacterial strain was determined by the induction with/without IPTG in the bacterial growth. YT10010(*sulA*) was the host and pMAL-c2X-*sulA*$^+$, pMAL-c2X-*sulA*(F143A), *sulA*(F143Y), *sulA*(F144N), *sulA*(M145I) were in series to transform the above strain; each of an individual transformant was expressing SulA and its derivatives by IPTG induction. (B) The degradation assays of MBP-SulA and its derivatives with ClpYQ protease. YT10010 (*sulA*) carrying pBAD24 and pBAD33 was transformed with pTH18kr-*mbp*, and the resultant transformant was used as the negative control. YT10010(*sulA*) carrying pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$ was separately transformed with pTH18-*mbp-sulA*$^+$ and its derivatives; the resulting transformants were grown with or without arabinose (0.5%) plus IPTG (the final concentration of 1mM) induction. The Western blot analyses indicated that the degradation of SulA or its derivatives by ClpYQ protease surveyed in the bacterial strain, using an anti-MBP anti-serum.

protein but had a certain binding affinity toward it (Van Melderen and Gottesman, 1999). As well, the deletion of C-terminal internal region of SulA does not have effects on the association between SulA mutants and Lon(S679A) (in S1 figure). However, using the yeast two-hybrid analysis, we have shown that SulAΔC20aa has higher affinity toward ClpY (M187I) (Fig. 2A). Yet, *in vivo* degradation assays indicated that SulAΔC20aa has a higher molecular weight in the Western blot analyses (Fig. 2F). These results suggest that removal of the final C-terminal 20aa of HA-SulA may lead to somewhat unfolding of HA-SulAΔC20aa molecules. Therefore, there is HA-SulAΔC20aa aggregate protein in the bacterial cells. However, it is still degraded by ClpYQ protease. In addition, in combination with ClpQ, we have used various ClpY mutants, which have mutation(s) in either the double loop of domain I or the pore I site, to degrade SulAΔC20aa in bacteria. The degradation patterns were similar to those observed using the full length HA-SulA as the substrates (data not shown). Besides, the bacterial cells carrying pMAL-c2X -*sulA*$^+$ plasmid can be induced by an IPTG induction and the resultant expression of MBP-SulA cause the cellular lethal (the data similar as that seen in Fig. 5A, lane 2). Interestingly, by contrast, the high copy plasmid pMAL-c2X carrying *sulA*ΔC20aa still produces active protein as the wild-type pMAL-c2X-*sulA*$^+$ in YT10010 (*sulA*) did after the IPTG induction (data not shown). Similarly, the rest pMAL-c2X-*sulA* C-terminal deletion derivatives in YT10010 (*sulA*) still maintained the subtle lethal activity (data not shown). These data suggest that the higher amount of SulA deletion mutant proteins in bacteria can compensate for their original defective activity in the lower amount. As well, both MBP-SulA and MBP-SulAΔC20aa can be degraded by ClpYQ protease *in vitro* assay (Hwang et al., 2014). Hence, the above results indicate that the last C-terminal 20 amino acid region of SulA molecule is not required for the substrate's recognition and degradation by ClpYQ protease in bacteria. Moreover, it has been shown before that SulA mutant protein with N-terminal deletion would not have effects on association with ClpY in the two-hybrid assays (Lee et al., 2003).

Subsequently, SulAΔC30aa and SulAΔC45aa did not interact substantially with ClpY(M187I) in the two-hybrid assay. Additionally, SulAΔC(20-45)aa and SulAΔC(20-30)aa did not well interact with ClpY (M187I). Furthermore, in the *in vivo* degradation assays, it showed that HA-SulAΔC(20～45)aa was not degraded by ClpYQ protease (Fig. 3E, lane 7 and 8). Additionally, HA-SulAΔC(20～30)aa was not fully degraded by the ClpYQ protease (Fig. 3E, lane 9 and 10). Therefore, SulA C-terminal internal region, 139th~149th aa(s), are necessary for the association with ClpY. As well, we found before that in the *in vitro* pull-down analyses, ClpY interacts with SulA and SulAΔC20aa. But, it was weakly interacted with SulACΔ30aa, SulACΔ45aa and SulAΔC(20～30) aa, respectively (Hwang et al., 2014). After using 2-fold concentration of Triton to wash off the complex of ClpY-SulA and its derivatives, ClpY can no longer associate with the above last three SulA deletion mutants (Hwang et al., 2014). These results indicate that all the above three SulA deletion mutants did not form a stable complex with ClpY. Therefore, ClpY was easily removed from the ClpY-SulA complex under a more stringent washing condition. In addition, using the hydroxyl-propensity prediction, SulA has excessive hydrophobic residues [8 aa(s) in 11 aa(s)] around 139th to 149th aa. We also observed consensus sequences between the 142th to 147th residues (Fig.1B). By mutagenesis of the certain amino acid residues in this region, as compared to the wild-type SulA, SulA(R146 L) has an equal binding affinity towards ClpY(M187I). In contrast, SulA(M145I) has a higher binding affinity toward ClpY(M187I). These results indicated that amino acid substitution from hydrophilic residue(s) to hydrophobic residue(s) would increase the binding affinity with ClpY(M187I). By contrast, SulA(F143Y) and SulA(I144 N), whose amino acid residue was changed from the hydrophobic to the hydrophilic characteristics, and these two mutant proteins have lower binding affinity toward ClpY(M187I). However, SulA(F143A) has only a subtle reduced binding affinity toward ClpY(M187I). The reason for this subtle alternation is unknown; one likely explanation could be alanine carries no strong hydrophilic groups. Taken together, this C-terminal internal region of SulA appears to be important for an association with ClpY. Likely, ClpY associates

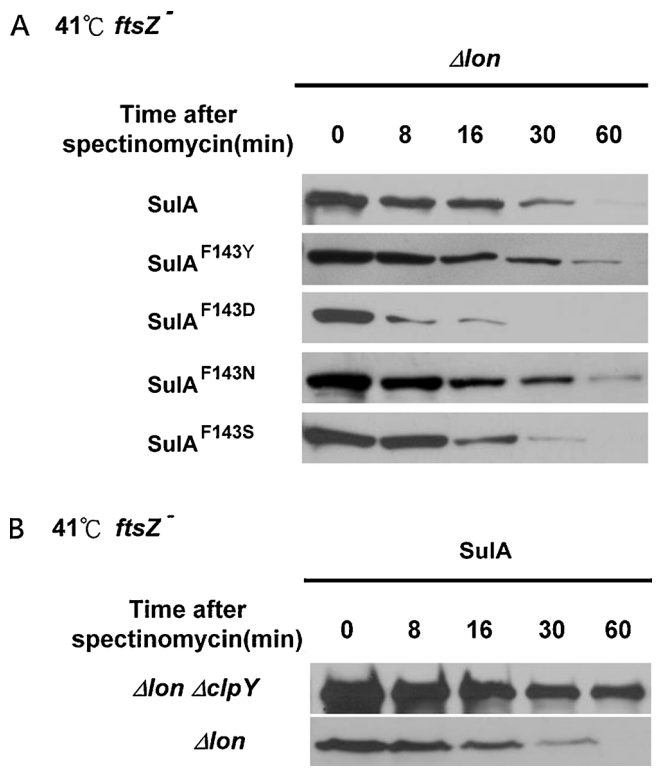

Fig. 6. The degradation of MBP-SulA and its single point mutation derivatives by ClpYQ protease without the Lon, FtsZ protein in the bacteria. (A) HT1004 (*lon, ftsZ*) mutant strain was transformed with pTH18-*mbp-sulA*$^+$ and its derivatives. The resultant each transformant was grown in LB media, with an appropriate antibiotic and an addition of IPTG (the final concentration of 1mM), to the OD600 = 0.5, separately. Subsequently, the spectinomycin (the final concentration 150 μg/ml) was added into the media at time zero, and the 1ml cells was taken for the preparation of the protein mixture. The procedures for preparation of protein samples at time intervals was as described in reference (Wu et al., 1999; Chang et al., 2016). The Western blot analyses was performed using an anti-MBP anti-serum. (B) The stable MBP-SulA in the bacteria HT1005 (*lon*, *clpY ftsZ*) mutant. Both HT1004 and HT1005 bacteria with pTH18-*mbp-sulA*$^+$ was treated as described above. The turn-over of MBP-SulA in both strains were determined.

with SulA through hydrophobic interactions.

Several other SulA point mutants, which had an amino acid alteration of 143th residue in the consensus region, were made by mutagenesis methods. It is interesting that ClpYQ degrades these different SulA point mutants with variously efficient activities. As compared, it has been shown that ClpYQ degraded the SulA (F143D) much more efficiently (Fig. 6A). ClpYQ degraded SulA(F143Y), SulA (F143N) and SulA(I144N) less efficiently (Figs. 5B and 6A). Accordingly, ClpY interacts with SulA well, but ClpY interacts loosely with SulA(F143Y) and SulA(I144 N) (Fig. 4A–C). These associations between ClpY and each of SulA point mutants have an effect on subsequent degradation by ClpYQ complex. However, it is likely that ClpY interacts with SulA(F143D) less efficiently due to the hydrophilic effects by amino acid alteration. By contrast, ClpYQ degraded SulA(F143D) more efficiently (Fig. 6A). It remains to be clarified for the reason with that ClpYQ degrades the SulA(F143D) so rapidly. One thought is that SulA(F143D) becomes a more suitable substrate for ClpYQ. ClpY can easily unfold/translocate its substrate, SulA(F143D), into the proteolytic core center for the degradation by ClpQ. In addition, in Fig. 6B, there is lower SulA level in Δ*lon* Δ*clpY* mutant at time 30 and 60 min and these data indicated that it is likely other proteases were also involved in the degradation of SulA itself.

Taken together, in this study, we demonstrated that a hydrophobic region, 142th ~ 147th residues, in the SulA C-terminally internal region is necessary for the recognition/degradation by ClpYQ protease. Moreover, in this region, SulA single point mutant(s) at position 143th residue could have effects on the recognition/binding and unfolding/translocation for the degradation by ClpYQ proteases.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.12.003.

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