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Characterization of the *Escherichia coli* ClpY (HslU) Substrate Recognition Site in the ClpYQ (HslUV) Protease Using the Yeast Two-Hybrid System[▽]

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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 the substrate-binding subunit. The ATP-dependent proteolysis is mediated by substrate recognition in the ClpYQ complex. ClpY has three domains, N, I, and C, and these domains are discrete and exhibit different binding preferences. In vivo, ClpYQ targets SulA, RcsA, RpoH, and TraJ molecules. In this study, ClpY was analyzed to identify the molecular determinants required for the binding of its natural protein substrates. Using yeast two-hybrid analysis, we showed that domain I of ClpY contains the residues responsible for recognition of its natural substrates, while domain C is necessary to engage ClpQ. Moreover, the specific residues that lie in the amino acid (aa) 137 to 150 (loop 1) and aa 175 to 209 (loop 2) double loops in domain I of ClpY were shown to be necessary for natural substrate interaction. Additionally, the two-hybrid system, together with random PCR mutagenesis, allowed the isolation of ClpY mutants that displayed a range of binding activities with SulA, including a mutant with no SulA binding trait. Subsequently, via methyl methanesulfonate tests and *cpsB*::*lacZ* assays with, e.g., SulA and RcsA as targets, we concluded that aa 175 to 209 of loop 2 are involved in the tethering of natural substrates, and it is likely that both loops, aa 137 to 150 and aa 175 to 209, of ClpY domain I may assist in the delivery of substrates into the inner core for ultimate degradation by ClpQ.

Escherichia coli ClpYQ (HslUV) is a two-component protease composed of ClpY (50 kDa) and ClpQ (19 kDa) (4, 12, 22, 28). ClpY is the regulatory subunit, with an ATPase, and has specific substrate-binding activities; ClpQ is the catalytic subunit, with peptidase activity (12, 22, 28). ClpQ and ClpY oligomerize as hexamers, and a two-tier stack of ClpQ and ClpY constitutes the protease (12, 22, 28). ClpY binds, unfolds, and transfers the substrates outside the cylinder into a catalytic core, where ClpQ degrades the substrates (5). Three cellular proteins are distinctively regulated by ClpYQ: SulA, a cell division inhibitor; RcsA, a positive regulator of capsule transcription; and RpoH, a heat shock sigma transcriptional factor (10, 11, 13, 15, 23, 24, 31, 33, 37). Recent studies have demonstrated that Cpx-mediated TraJ degradation by ClpYQ is stress dependent (18). Additionally, the basal and heat shock induction of the *clpQ*$^+$ *clpY*$^+$ operon is mediated solely by the RpoH factor (4, 20), and the stem-loop structure in the 5′ untranslated region of *clpQ*$^+$ *clpY*$^+$ mRNA(s) was shown to be necessary for the stability of its transcript(s) (20).

The ClpY molecule is divided into three domains: the N-terminal domain (N; residues 2 to 109 and 244 to 332), the I-intermediate domain (I; residues 110 to 243), and the C-terminal domain (C; residues 333 to 443) (2) (Fig. 1). The N domain has an ATPase activity (Fig. 1), and the C domain is responsible for self-oligomerization of ClpY (2). Based on X-ray structural analysis, it has been proposed that the I domain of ClpY is responsible for association with ClpQ (2). Therefore, the observed mode suggests that there is not a necessity for translocation of the substrate through the rather small central core of the ClpYQ hexamer; moreover, ClpY might deliver substrates through an altered active state of affinity for ClpQ (2, 27, 33). In contrast, based on electron microscopy images, in the docking mode, domain I appears to protrude from the ClpYQ complex with the process of substrate delivery unresolved (9, 29, 36). To investigate the above discrepancy, a deletion mutant, ClpY[Δ(175-209)] was made and was shown to be incapable of degrading the MBP-SulA fusion protein in the presence of ClpQ (33). However, the molecular mechanism for this observation remains unclear. In addition, in a biochemical study, the C-terminal tail was capable of stimulating ClpQ proteolytic activity (27, 30), and the G^{90} Y^{91} V^{92} G^{93} pore motif of ClpY was involved in the unfolding and translocation of protein substrates into the inner core for degradation by ClpQ (26, 40).

Notably, ClpY interacts with SulA in the two-hybrid assay (19). Also, in an analysis by real-time monitored surface plasmon resonance, it has been demonstrated that ClpY increases fivefold in binding affinity toward ClpQ while it is associated with MBP-SulA (1). However, deletion of the Thr active site in ClpQ also increases the binding affinity of its subunits with ClpY(s) (25). Using a phage 22 Arc repressor, an artificial substrate, the bound ATP in hexameric ClpY(s) is required for

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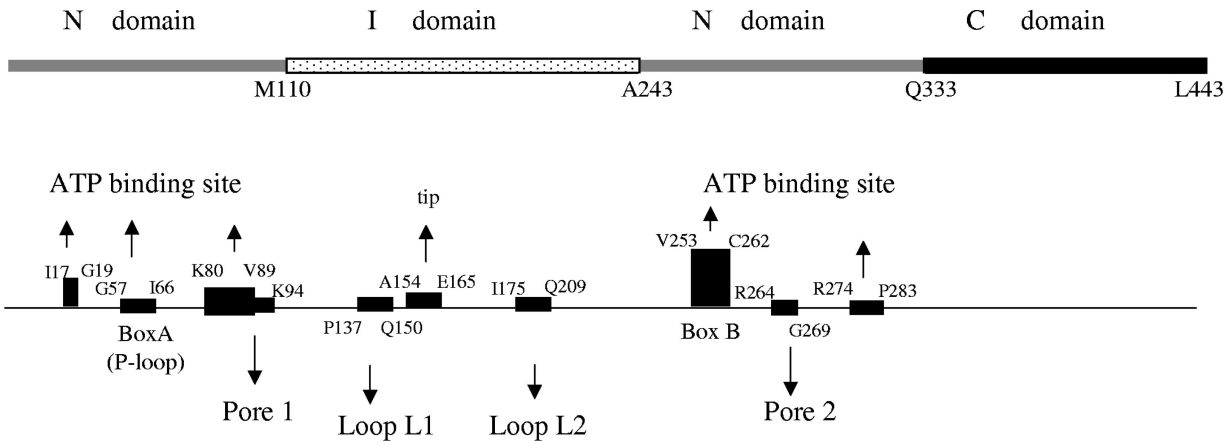


The selective ClpY loop mutants or those made by the site-directed mutagenesis

a L1 loop, 137-150 aa; a L2 loop, 175-209 aa								
I186N	M187I	A188S	L199Q	N205K	E193L, E194L	Q198L, Q200L	N141L, N142L	Q148L, Q149L, Q150L
ATT AAT	ATG ATT	GCT TCT	CTG CAG	AAC AAA	GAA, GAG CTA, CTG	CAG, CAG CTG, CTG	AAC, AAC C TC, CTC	CAG, CAG, CAG CTG, CTG, CTG

FIG. 1. Domains of ClpY and its functional motifs (top), as well as its derivative mutants (bottom). (Top) The total 443 aa (1,332 bp) of ClpY includes domains N, I, and C. Notable features include ATP binding sites; the Walker BoxA, or P loop (aa 57 to 66), and BoxB (aa 253 to 256 aa); the nucleotide binding pocket (aa 17 to 19, aa 57 to 66, and aa 80 to 89); the domain I tip (aa 154 to 165) and two loops (aa 137 to 150 and aa 175 to 209); and pore I (aa 89 to 94) and pore II (aa 264 to 269). (Bottom) The selective ClpY mutants and the site-specific substituted ClpY mutants (top row). The original nucleotides of the amino acids (middle row) and those of the substituted mutants (bottom row) are shown.

substrate recognition (3). Additionally, asymmetric binding and hydrolysis of ATP(s) were demonstrated in vitro for degradation of the substrates in the ClpYQ complex (39). The physiological role and biochemical function in eubacteria or in higher organisms of ClpYQ protease were recently reviewed by Wu et al. (38).

It is therefore of interest to determine how ClpY recognizes its natural substrates at the molecular level. Here, using the yeast two-hybrid approach, we show that domain I of ClpY is responsible for the recognition of its natural substrates while domain C is necessary for association with ClpQ. The amino acid (aa) 175 to 209 loop plays a role in substrate fastening. We further identified two specific loops (aa 137 to 150 and aa 175 to 209) that together, in domain I of ClpY, are required for association with its natural substrates. In addition, our results suggest that both loops, aa 137 to 150 and aa 175 to 209, of ClpY are also likely involved in substrate delivery for degradation by ClpQ.

MATERIALS AND METHODS

Strains and plasmids. The yeast *Saccharomyces cerevisiae* EGY48 [*MAT***a** *his3 trp1 ura2 lexA*$_{op(x6)}$*-leu2*] reporter strain, which carries p8op-*lacZ* plasmids and *lexA*$_{op(x6)}$*-LEU2* integrated into the chromosome, was obtained from Clontech (Palo Alto, CA). *E. coli* strain KC8 (*leuB trpC hisB*) and pGilda, a LexA DNA binding domain (BD) vector, were obtained from Clontech. Plasmid pB42AD, a B42 polypeptide activation domain (AD) vector with a hemagglutinin (HA) epitope tag, was also purchased from Clontech. *E. coli* AC3112 (*lon* Δ*clpQY cpsB*::*lacZ*) (15) was used as a host for the methyl methanesulfonate (MMS) test and the β-galactosidase assay for *cpsB*::*lacZ* expression. The plasmids pB42AD-*clpY*$^+$, pB42AD-*sulA*$^+$, pB42AD-*sulA*(*M89I*), pGilda-*sulA*$^+$, and pGilda-*sulA*(*M89I*) were prepared as described previously (19). Plasmids pBAD33 and pBAD24 for gene cloning were under arabinose induction or glucose repression (6).

Media and reagents. *E. coli* was grown in Luria-Bertani (LB) broth. For yeast growth, yeast-peptone-dextrose and synthetic dropout (DO) minimal medium (SD) were purchased from Clontech. DO supplements containing nucleotides and amino acid residues were prepared according to the manufacturer's instructions. Minimal media with different sugars were prepared as described by Miller (21). Additional supplements were added at the following stock concentrations: glucose, 2%; galactose (Gal), 2%; raffinose (Raf), 1%; ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 34 μg/ml; and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 80 μg/ml.

PCR randomized mutagenesis in the domain I locus and selection of ClpY mutants for altered SulA binding in yeast two-hybrid assays. Mutations in DNA fragments of ClpY domain I were generated by error-prone PCR amplification of the target region from pB42AD-*clpY*$^+$ using power *Tag* DNA polymerase (GeneTeks, Taipei, Taiwan) under reaction conditions that favored the incorporation of mutations (i.e., using Mn^{2+} instead of Mg^{2+}). The oligonucleotide primers for PCR randomized amplification of domain I were as follows: forward primer, 5′-AAAACTGAAATCGCCCGTCG-3′, and reverse primer, 5′-CAGC AGGTCACGCTGAACGC-3′. The two primers were annealed to the *clpY*$^+$ plasmid template for amplification of DNA sequences from the nucleotides of aa 63 to the end of aa 282 of ClpY. PCR products that encompassed the region of domain I were therefore made for homologous recombination when cotransformed with linearized pB42AD-*clpY*$^+$ (cut with NruI in the *clpY*$^+$ gene) into EGY48 carrying pGilda-*sulA*$^+$. This method has been described previously (7). Recombinants were then selected by growing the yeast on SD lacking uracil, tryptophan, and histidine (SD −Ura −Trp −His). The interaction between ClpY mutants and SulA was tested by scoring the resulting transformants of each on

X-Gal minimal medium with the addition of Raf and Gal. White or dark-blue colonies were saved for further isolation of plasmids. The extracted plasmids were subsequently transformed into *E. coli* KC-8, and the resultant transformants were selected for Trp^+ on glucose minimal medium plus DO without the addition of tryptophan. pB42AD plasmids carrying *clpY*$^+$ and its mutant derivatives were recovered from Trp^+ colonies. Plasmids that expressed full-length *clpY*$^+$ and its derivatives were verified again after retransformation into EGY48(p8op-*lacZ*) carrying pGilda-*sulA*$^+$ by scoring their new transformants on X-Gal medium and testing them on a Leu-depleted plate. The isolated plasmids, with no autoactivation of the reporters in the yeast, were then subjected to DNA-sequencing analysis to determine the site of the mutation(s).

To assess the expression of the reporters, the yeast strain EGY48(p8op-*lacZ*) coexpressing BD and AD hybrid proteins was then subjected to β-galactosidase analysis and scored on an X-Gal plate, in addition to the *leu*$^+$ selective assays using methods described previously (19). Each assay was performed at least three times, and each time, the β-galactosidase analysis was performed in triplicate for the yeast cells.

Construction of plasmids and site-directed mutagenesis of ClpY mutants. PCR was used to amplify the desired gene using MG1655 chromosomal DNA as the template with its DNA extraction as described by Silhavy et al. (32) or by using *clpY*$^+$ *clpQ*$^+$ plasmids or their derivatives as the templates as described previously (19). However, plasmid pGilda-*clpYX* was constructed using two-step PCR amplification. The forward primer, F1 (5′-CGCGGATCCATATGTCTG AAATGACCCCA-3′), with a BamHI cutting site (underlined), and the reverse primer, R1 (5′-ATCAGAGCTTCTTCGCTCCAGTTCAACGCGGATTGG-3′), were used for amplification of a portion of the *clpY*$^+$ gene encoding aa 1 to 333. The other primer set, F2 (5′-CCAATCCGCGTTGAACTGGAGCGAAG AAGCTCTGAT-3′) and R2 (5′-CCGCCGCTCGAGTTATTCACCAGATGC CTG-3′), with an XhoI site (underlined), was used to amplify part of the *clpX*$^+$ gene encoding the C-terminal domain from aa 319 to 424. After Gel-M (Viogene, Taipei, Taiwan) purification, these two PCR products were then taken as the template for second-round PCR amplification using F1 and R2 as the primers; the resulting PCR products were purified and digested with the appropriate restriction enzymes, and the purified product was cloned into pGilda at the BamHI-XhoI sites. To construct pB42AD-*clpYX*, we cloned the PCR product of the *clpYX* hybrid gene into pB42AD at the XhoI site by using 5′-CCCCGCTC GAGATGTCTGAAATGACCCCA-3′ (F3), with an XhoI site (underlined), and the R2 reverse primer amplified from pGilda-*clpYX*. The *clpYX* gene was inserted with the correct orientation and was in frame with the HA tag. The plasmids pGilda-*clpY*(Δ*L1*) (aa 137 to 150 deleted), -*clpY*(Δ*L2*) (aa 175 to 209 deleted), -*clpY*(Δ*L1*, Δ*L2*) (aa 137 to 150 and aa 175 to 209 deleted), and -*clpY* (Δ*I*+*7Gly*) (with domain I deleted but with a seven-Gly insertion)] were constructed by two-step PCR methods. We used primer F4 (5′-CCGGAATTCAT GTCTGAAATGACCCCA-3′) (the EcoRI site is underlined) and R3 (5′-CTT TTTTGCTTCTCGATTTCTTTGTC-3′) for the front DNA fragment of *clpY* (Δ*L2*) and F5 (5′-AGAAATCGAGAAGCAAAAAGCGCGTAAG-3′) and R4 (5′-CCCGCGGATCCTTATAGGATAAAACGGCTC-3′) (the BamHI site is underlined) for the rest of the DNA fragment for *clpY*(Δ*L2*). The resulting two PCR fragments were then used as templates for F3 and R4 amplification. The resulting DNA fragment encoding ClpY(ΔL2) was cloned into pGilda at the EcoRI and BamHI sites. The resulting plasmid was designated pGilda-*clpY* (Δ*L2*). A similar two-step PCR method was used to construct plasmids *clpY* (Δ*L1*) and *clpY*(Δ*I*+*7Gly*). For *clpY*(Δ*L1*), we used the F3 primer and the R5 reverse primer (5′-GACGGTTCGATCAGCACGTCGAGAATAC-3′) for the front portion, as well as the F6 primer (5′-CGTGCTGATCGAACCGTCCGC TGCTCGTCAG-3′) and R4 for the remaining portion. For *clpY*(Δ*I*+*7Gly*), we used the internal primer R6 (5′-CCACCGCCACCGCCACCGCCTTTCACGG CGGCATCG-3′) and the F3 primer for the front portion and F7 (5′-GCGGT GGCGGTGGCGGTGGCATCGACGCTGTTGAGCAG-3′ and the R4 primer for the remaining portion. Plasmid pGilda-*clpY*(Δ*L1*, Δ*L2*) was constructed using pGilda-*clpY*(Δ*L2*) as the template for a two-step PCR amplification with the two pairs of primers (F4/R5 and F6/R4) listed above. The full-length DNA was then amplified by the F4 and R4 primers. The pGilda-*clpY*$^+$ (SmaI) plasmid was constructed by introducing an SmaI site into the middle of the *clpY*$^+$ gene (CC**T** CCG GGC→CCC CCG GGC [with a new SmaI site underlined]) (aa 189, 190, and 191) with no alterations in the encoded residues. This was accomplished by cloning the front portion of the *clpY* gene, encoding aa 1 to 190 (with EcoRI-SmaI sites at the ends) and the rest of the gene encoding aa 191 to 443 (with SmaI-BamHI sites at the ends) into pGilda. The plasmid pB42AD-*clpY*$^+$ (SmaI) was constructed by subcloning EcoRI-XhoI *clpY*$^+$ (SmaI) from pGilda-*clpY*$^+$ (SmaI) into pB42AD. Plasmids pB42AD-*clpY*(*E193L*, *E194L*) and -*clpY*(*Q198L*, *Q200L*) are derivatives of *clpY*$^+$ (SmaI). Plasmids pB42AD-*clpY*(*N141L*, *N142L*) and -*clpY*(*Q148L*, *Q149L*, *Q150L*) were also constructed by two-step PCR with the mutagenic primers using EcoRI and XhoI cloning sites at the ends to generate the mutated nucleotides of the amino acid substitutions in *clpY*$^+$. Substitutions of the nucleotides in *clpY*$^+$ or *clpY*$^+$ (SmaI) by mutagenesis were verified by DNA sequencing and are shown in Fig. 1. The other plasmid, pGilda-*clpY*$^+$, was used as described in a previous study (19). The plasmids pB42AD-*clpY*(Δ*L1*), pB42AD-*clpY*(Δ*L2*), pB42AD-*clpY*(Δ*L1*, Δ*L2*), and pB42AD-*clpY* (Δ*I*+*7Gly*) were constructed by cutting the 1.2-kb DNA fragments out of the four corresponding plasmids separately and individually ligating all four fragments into pB42AD at the EcoRI-XhoI sites, after which the procedures for cloning were as described above.

PCR amplification of the *clpQ*$^+$ gene, including the upstream 63-bp untranslated leader sequences, was performed using the ClpQ N-terminal primer 5′-G AATTCCAGCTCGGTACCGCATTATGCCCCGTA-3′ and the ClpQ C-terminal primer 5′-GGGGCTGCAGTTACGCTTTGTAGCT-3′ (the restriction enzyme sites are underlined). The PCR amplicons were cloned into pBAD33 KpnI-PstI sites, and the resulting plasmid was designated pBAD33-*clpQ*$^+$. Plasmid pBAD24-*clpY*$^+$ and its derivatives were constructed by cloning *clpY*$^+$ and its derivatives, about 1.2 kbp starting from the ATG codon and ending at the stop codon, into pBAD24 at EcoRI-HindIII sites. Additionally, pBAD24-*clpYX* was constructed by cloning the smaller NdeI-XhoI fragment from pGilda-*clpYX* into the NdeI-HindIII sites of pBAD24-NdeI (a construct that generates a new NdeI site between the EcoRI and NcoI sites of pBAD24) with a correct insert. Correct insertions in all the above pBAD24-*clpY*$^+$ and derivative plasmids were confirmed by restriction enzyme mapping.

MMS test and β-galactosidase assays for *cpsB*::*lacZ* expression. *E. coli* AC3112 cells (*lon* Δ*clpQY cpsB*::*lacZ*) (15) carrying the plasmid pBAD33-*clpQ*$^+$ in combination with pBAD24-*clpY*$^+$ or with mutant *clpY*-relevant plasmids were grown overnight on LB agar with ampicillin (50 μg/ml) and chloramphenicol (17 μg/ml), as well as glucose (1%) or arabinose (0.5%). One percent of the overnight culture was inoculated into fresh medium with the same ingredients, and the cell culture was grown to log phase. The cells were then immediately subjected to the MMS test, as well as the β-galactosidase assay. An 8-μl sample of a serial dilution of the log-phase cells was spotted onto LB agar-MMS plates (0.025% MMS) and LB agar with the addition of glucose (1%) or arabinose (0.5%). The efficiency of plating (EOP) was calculated by comparing the titers of colonies on LB agar medium with added MMS and arabinose divided by those of colonies on LB agar plus arabinose. Half of the log-phase cells were subjected to the β-galactosidase assay in triplicate, using Miller units (21).

Construction of HA-*sul*A$^+$ in a single-copy plasmid under a *tac* promoter and Western blot assay of bacterial cells. The plasmid pTHkr18-*ha-sulA*$^+$ was constructed by first using the plasmid pB42AD-*sulA*$^+$ as a template for amplification of the HA-*sulA*$^+$ fragment, since pB42AD-*sulA*$^+$ carries an in-frame fusion of an HA tag in front of *sulA*$^+$. The forward primer 5′-GTACCCATATGTACCCTT ATGATGTGCCAGATTAT-3′ and the reverse primer 5′-GGGGGATCCGGA TCCTTAATGATATACAAATTAGAGTG-3′ (the restriction enzyme sites are underlined) were used for the PCR amplification. The resulting DNA fragment was cloned into the plasmid pTHkr18 (8) at the NdeI and BamHI sites. Therefore, plasmid pTH18kr-*ha-sulA*$^+$ contains HA-*sulA*$^+$ under *tac* promoter regulation, and AC3112 carries a *lacI*^{q} marker. AC3112 variants carrying pBAD33-*clpQ*$^+$ (Chl^r) with a combination of pBAD24-*clpY*$^+$ (Amp^r) or its derivative plasmids were each transformed with pTH18kr-*ha-sulA*$^+$ (Kan^r), and the colonies were selected on LA plates with ampicillin (50 μg/ml), chloramphenicol (17 μg/ml), and kanamycin (12.5 μg/ml). AC3112 cells simultaneously containing pBAD33, pBAD24, and pTH18kr-*ha-sulA*$^+$ were used as a control. Next, AC3112 cells carrying pTH18kr-*ha-sulA*$^+$, pBAD33-*clpQ*$^+$, and either pBAD24-*clpY*$^+$ or its *clpY*$^+$ derivatives were grown in series overnight on LB medium with 0.5% arabinose and the appropriate antibiotics. Dilutions (1:100) of the overnight bacterial cultures were inoculated in fresh medium and were grown at 30°C. After 3 hours of growth to an optical density at 600 nm (OD_{600}) of 0.5, 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each culture at a final concentration of 1 mM. After a 2- or 3-hour interval, 1-ml aliquots were taken, and the OD_{600} was recorded. Cellular extracts were then collected and separated on 12.5% sodium dodecyl sulfate-polyacrylamide gels (17). Western blotting was performed using a monoclonal HA antibody (Roche) to detect HA-SulA and polyclonal ClpQ and ClpY antibodies for the detection of the original protein molecules. The Western blots were developed using enhanced chemiluminescence (Pierce).

RESULTS

Based on the X-ray crystal structures (2) and electron microscopy analysis (9), as well as the investigation of *Haemophi-*

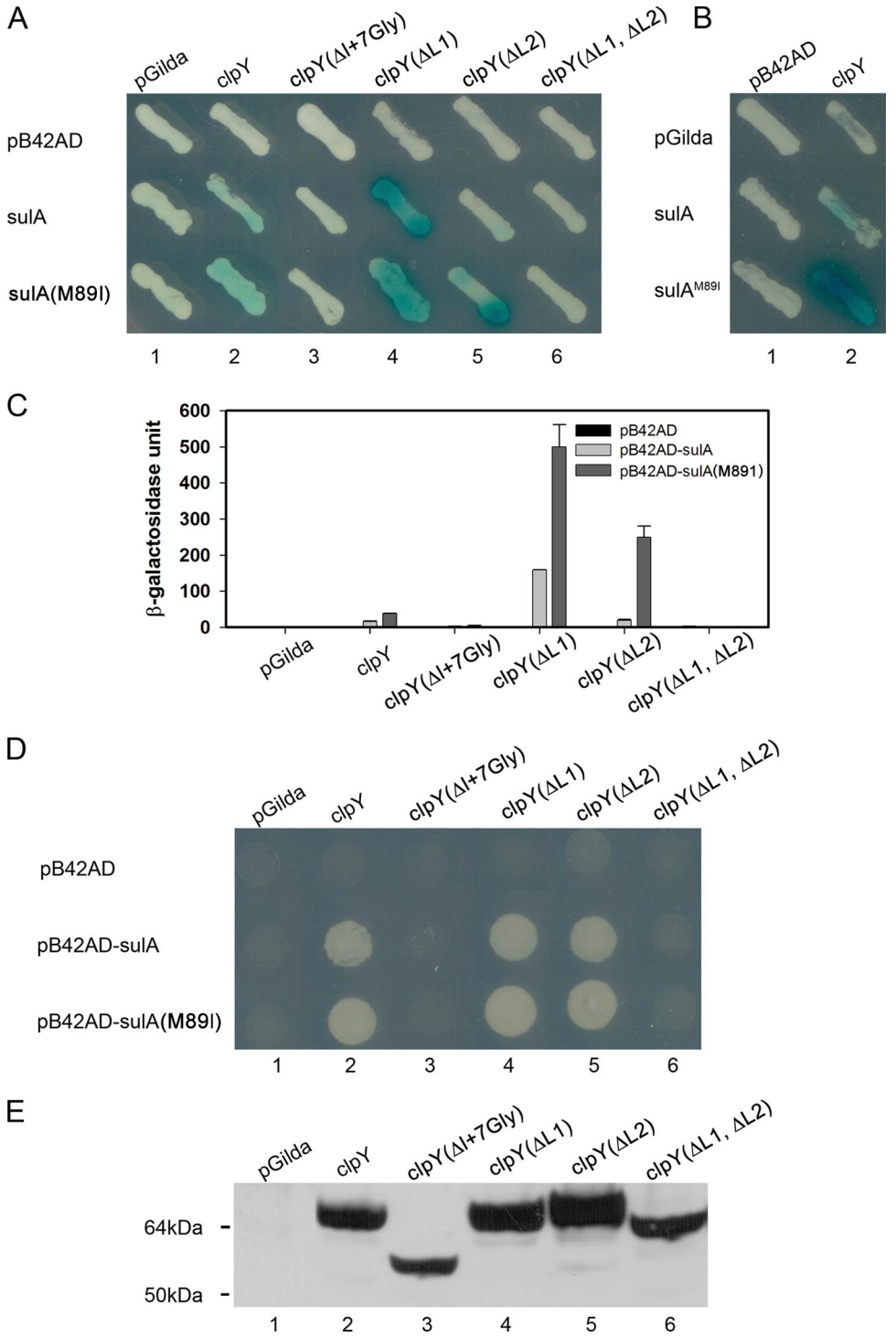


FIG. 2. Expression of *lacZ* and LEU2 in EGY48(p8op-*lacZ*), each with a pair of AD and BD fusion proteins. The BD is from pGilda, and the AD is from pB42AD. Each fusion protein is indicated. (A) Expression of *lacZ*, with the colony color evaluated on Gal +Raf −Ura −His −Trp plates containing X-Gal over 4 days. The top row is the control, in which yeasts express BD-ClpY or its derivative mutants with an AD. The middle row is BD-ClpY and its derivative mutants with AD-SulA. The bottom row is ClpY and its derivative mutants with SulA(M89I). (B) Lane 1, reciprocal negative control; lane 2, AD-ClpY with BD-SulA/SulA(M89I). (C) β-Galactosidase activities of yeasts carrying BD-ClpY or its derivatives, AD-SulA or SulA(M89I). The error bars indicate standard deviations. (D) LEU2 expression of yeasts on Gal +Raf −Ura −His −Trp −Leu plates over 4 days. The top row is the control, as described for panel A. The middle row shows yeasts, grown on *leu*-deficient plates, expressing BD-ClpY or its derivative mutants with AD-SulA. The bottom row shows yeasts expressing BD-ClpY or its derivative mutants with AD-SulA(M89I). (E) Western blotting of ClpY and its derivatives in the BD fusion protein using an anti-LexA antibody.

lus influenzae (35), questions were raised about whether the *E. coli* ClpYQ complex has the I domain of ClpY oriented toward or away from the ClpQ hexamer. Previously, we demonstrated that ClpY (fused with an AD) interacts with SulA (fused with a BD) in the yeast-two hybrid assays (19). We showed that an artificial transcriptional factor was reconstituted when the yeast EGY48(p8op-*lacZ*) expressed both AD-ClpY and BD-SulA. When both ClpY and SulA were associated with each other under induction by the addition of two sugars, Raf and Gal, the *lacZ* and *leu2* genes were activated. Yeast transformants were therefore grown on *leu*$^+$ selective medium (SD plus two sugars without the addition of leucine) and were light blue on X-Gal medium. In this study, using the yeast two-hybrid approach (also with a reciprocal combination of BD-ClpY and AD-SulA), we began to search for a functional region of ClpY that is involved in recognition of the natural substrates and a functional domain of ClpY that forms an association with ClpQ.

There is no interaction of ClpY(ΔI+7Gly) and ClpY(ΔL1, ΔL2) with SulA in the two-hybrid assay. To determine whether the I domain of ClpY is responsible for the recognition of substrates, we made a *clpY*(Δ*I +7Gly*) gene, with the domain I gene deleted in *clpY*$^+$ but with seven glycine codons inserted at a site between domains N and C. This deletion mutant has a configuration similar to that of ClpY on analysis of its X-ray crystal structure (16). This DNA fragment was cloned into the pGilda vector, and the resulting plasmid was designated pGilda-*clpY*(Δ*I+7Gly*). This plasmid was thereafter transformed into the yeast EGY48(p8op-*lacZ*) carrying pB42AD-*sulA*$^+$, and the resultant transformants were selected on SD −Ura −Trp −His. The yeasts carrying BD-ClpY(ΔI+7Gly)/AD-SulA were white on X-Gal plates, with lower β-galactosidase activities, and did not grow on *leu*-deficient medium (Fig. 2A, C, and D, lanes 3); these results were similar to those with the negative control cells (Fig. 2A, C, and D, lanes 1) carrying pGilda and pB42AD-*sulA*$^+$. Therefore, no interaction was observed between ClpY(ΔI+7Gly) and SulA. In contrast, yeasts with BD-ClpY/AD-SulA or BD-SulA/AD-ClpY as a separate positive control were light blue, had a subtle increase in β-galactosidase activity, and appeared Leu$^+$ on the selective media (Fig. 2A, C, and D, lanes 2; compare B, lane 2, to lane 1). When SulA(M89I) was instead expressed in yeasts, it exhibited enhanced interaction with ClpY [dark blue in yeast with BD-SulA(M89I)/AD-ClpY (Fig. 2B, lane 2) or with BD-ClpY/AD-SulA(M89I) (Fig. 2C, lane 2)] (19), and these results were similar to those for the yeast strains in which SulA was expressed (Fig. 2A, C, and D, lanes 2 to 6).

Accordingly, the ClpY[Δ(137-150)] mutant, carrying a deletion of aa 137 to 150, maintained the most activity for degradation of MBP-SulA in the presence of ClpQ (33). In contrast, a ClpY(ΔL2) mutant, with a deletion of aa 175 to 209, apparently lost its ability to have the substrate degraded by ClpQ (33). The reason for this observation remains unclear. Thus, we investigated whether these double loops in domain I of ClpY have functional activity in regard to recognition of the substrates. Two mutants with a single-loop deletion, *clpY*[Δ(137-150)] and *clpY*[Δ(175-209)], were made in series and then were individually cloned into pGilda, and the resulting plasmids were designated pGidla-*clpY*(Δ*L1*) and pGilda-*clpY*(Δ*L2*), respectively. A double-loop deletion, *clpY*[Δ(137-150), Δ(175-209)], was made as well and cloned into pGilda, and the resulting plasmid was designated pGilda-*clpY*(Δ*L1*, Δ*L2*). Each plasmid was cotransformed with pB42AD-*sulA*$^+$ into the yeast EGY48(p8op-*lacZ*), and the resultant cotransformants, carrying the plasmid pair, were scored on X-Gal plates and tested on *leu*-deficient medium. Yeasts carrying ClpY(ΔL1, ΔL2)/SulA were white on X-Gal plates, with lower β-galactosidase activities, and also did not grow on *leu*-deficient medium (Fig. 2A, C, and D, lanes 6). Thus, the ClpY (ΔL1, ΔL2) molecule was not associated with SulA/SulA(M89I). However, ClpY(ΔL1) or ClpY(ΔL2) significantly interacted with SulA/SulA(M89I) (Fig. 2A, C, and D, lanes 4 and 5). The yeast colonies carrying BD-ClpY(ΔL1)/AD-SulA [SulA(M89I)] had much higher β-galactosidase activities than the yeast strains carrying BD-ClpY(ΔL1, ΔL2)/AD-SulA [SulA(M89I)] or BD-ClpY(ΔL2)/AD-SulA [SulA(M89I)] (Fig. 2A and C, lanes 4, 5, and 6). Therefore, aa 175 to 209 of ClpY are likely involved in an enhanced binding of the SulA molecule.

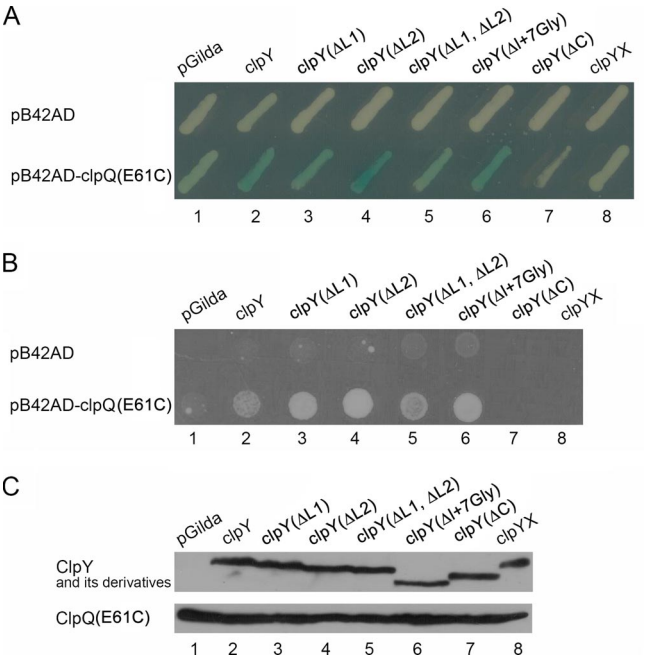


FIG. 3. Expression of *lacZ* and LEU2 in EGY48(p8op-*lacZ*) cells carrying ClpY and its derivative mutants with ClpQ(E61C). (A) The top row shows the control yeasts on X-Gal plates over 4 days. The bottom row illustrates the interaction between ClpY and its derivative mutants with ClpQ(E61C). (B) LEU2 expression of yeasts on Gal +Raf −Ura −His −Trp −Leu plates over 4 days. The top row is the control. The middle row shows each strain carrying BD-ClpY or its derivative mutants with AD-ClpQ(E61C). (C) Western blotting of ClpY and its derivatives and ClpQ(E61C) using anti-LexA and anti-HA antibody, respectively.

Western blotting with the polyclonal LexA antibody was used to detect ClpY and its derivative mutants in the yeast cells. The LexA-ClpY fusion protein and its derivatives were detected as shown in Fig. 2E.

ClpY(ΔL1), ClpY(Δ L2), ClpY(ΔL1, ΔL2), and ClpY (ΔI+7Gly) associated with ClpQ(E61C), but not ClpY(ΔC) and ClpYX. We also tested the interaction of the wild-type ClpY and the ClpY derivative mutants ClpY(ΔL1), ClpY(Δ

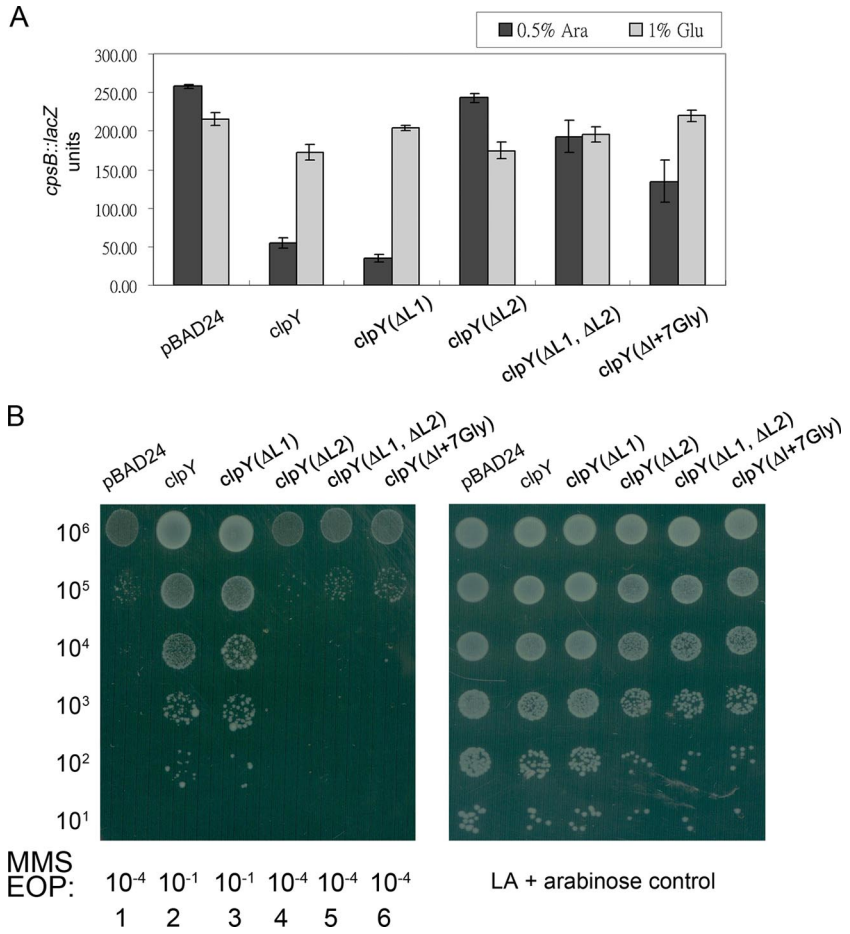


FIG. 4. Chart of *cpsB*::*lacZ* expression and MMS tests for each bacterial-cell condition. The AC3112 strain carrying pBAD24-*clpY*$^+$ or its derivatives with pBAD33-*clpQ*$^+$ was grown in LB broth with appropriate antibiotics and 0.5% arabinose (as an induction) or 1% glucose (as a repressed condition) to an OD_{600} of 0.5 to 0.9, and bacterial samples from each were removed for measurement of β-galactosidase activity (A) and MMS tests (B). Serial dilutions of the bacterial cultures were spotted on LB plates plus 0.5% arabinose, with an addition of 0.025% MMS. The EOP value was taken as the average number of colonies formed on medium with 0.025% MMS divided by the average number of colonies detected on medium without the addition of 0.025% MMS. Bacterial cells carrying pBAD33 and pBAD24 were used as negative controls.

L2), ClpY(ΔL1, ΔL2), and ClpY(ΔI+7Gly) with ClpQ(E61C). E61-ClpQ is bridged with R440-ClpY in the X-ray crystal structure (29). ClpQ(E61C) not only has higher affinity for ClpY, it also retains normal proteolytic activity (our unpublished data). pGilda-*clpY*$^+$, -*clpY*(Δ*L1*), -*clpY*(Δ*L2*), -*clpY*(Δ*L1*, Δ*L2*), and -*clpY*(Δ*I*+*7Gly*) were separately transformed into yeast carrying pB42AD-*clpQ*(*E61C*), and all the resultant transformants were selected on SD −Ura −Trp −His. These yeast cotransformants were then tested on *leu*-deficient medium and streaked on X-Gal medium. The transformants were blue on X-Gal plates and grew well on the *leu*-deficient medium. As shown (Fig. 3A and B, compare lanes 3, 4, 5, and 6 to lane 2), BD-ClpY(ΔL1), -ClpY(ΔL2), -ClpY(ΔL1, ΔL2), and -ClpY(ΔI+7Gly) were each associated with AD-ClpQ(E61C) in the two-hybrid assays, as was seen with the wild-type ClpY. To determine whether the C domain of ClpY is responsible for the association with ClpQ, the plasmid pGilda-*clpYX*, encoding the chimeric protein ClpYX, was constructed using two-step PCR. Notably, ClpX did not associate with ClpQ; it formed a hexamer, and its C domain (from aa S318 to E424) had ~41% similarity to the ClpY counterpart (from aa T336 to L443)

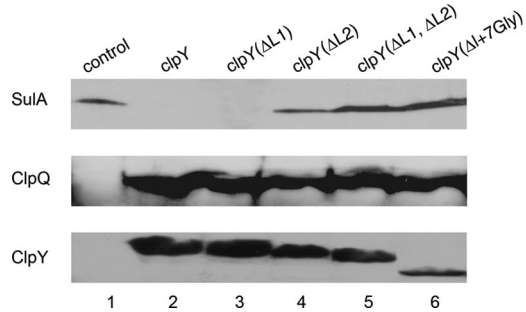


FIG. 5. Residual HA-SulA and ClpQ, as well as ClpY and its derivatives, for each bacterial strain. Cells were grown to an OD_{600} of 0.3 in LB broth with the addition of appropriate antibiotics and 0.5% arabinose; then, 1 mM IPTG was added to the medium for induction of HA-SulA for 2 h, after which samples were extracted, normalized to the OD_{600}, and loaded as described in Materials and Methods. The top row shows the residual SulA, the middle row shows ClpQ, and the bottom row shows ClpY and its derivatives. The anti-HA monoclonal antibody was used for HA-SulA, and the anti-ClpQ and anti-ClpY polyclonal antibodies were also used separately.

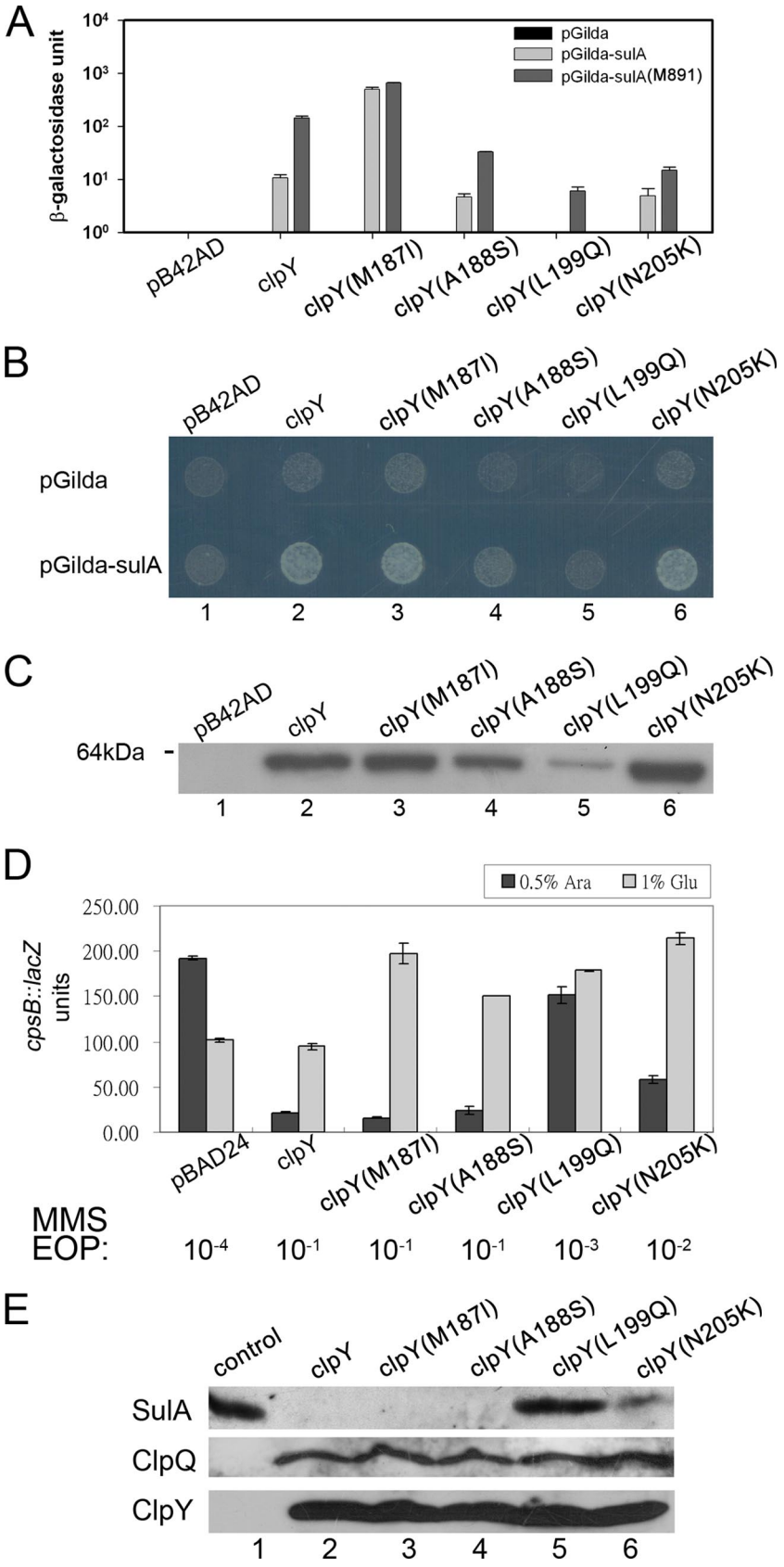


FIG. 6. (A) Assays of β-galactosidase activities in yeasts; each pair of AD or BD fusion proteins is indicated. The yeast EGY48(p8op-*lacZ*) carries ClpY or its derivative mutants in AD and SulA/SulA(M89I) in BD, separately. Shown is LacZ expression of the control, the AD-ClpY or

(14). Therefore, a *clpYX* plasmid, with N and I domains of ~333 aa encoded by the *clpY*$^+$ gene and the remaining 106 aa from the C domain (aa 319 to 424) of ClpX, was transformed into the yeast with pB42AD-*clpQ*(*E61C*). Similarly, pGilda-*clpY*(Δ*C*) (19) with a deletion of domain C, constructed previously, was also transformed into the yeast EGY48 carrying AD-ClpQ(E61C). The resulting transformants did not grow on *leu*-deficient medium and were white on X-Gal medium (Fig. 3A and B, lanes 7 and 8). These results indicate that neither ClpYX nor ClpY(ΔC) interacted with ClpQ(E61C). Western blot analysis using anti-LexA and anti-HA antibodies revealed that ClpY and its derivatives, as well as ClpQ(E61C), were appropriately expressed (Fig. 3C). Thus, these results suggest that the C domain of ClpY is directly responsible for association with ClpQ.

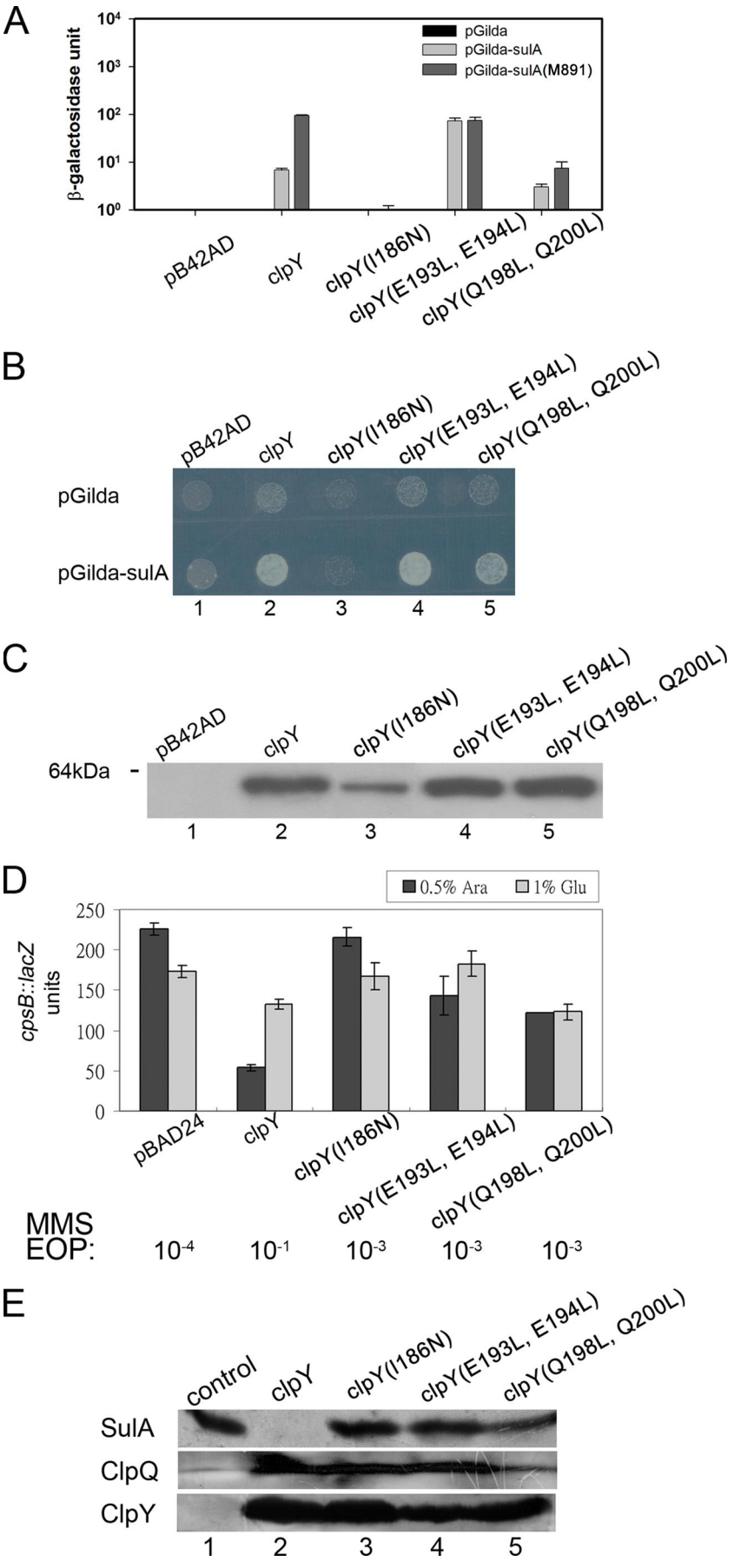
ClpY(ΔI+7Gly), ClpY(ΔL2), and ClpY(ΔL1, ΔL2) did not target the natural substrates in the presence of ClpQ. To investigate the intracellular activities of wild-type ClpY and its derivative mutants in bacteria, we subcloned *clpY*$^+$ and its derivative genes from their corresponding pB42AD derivative plasmids into pBAD24; the resultant plasmids were designated pBAD24-*clpY*$^+$, pBAD24-*clpY*(Δ*I+7Gly*), pBAD24-*clpY*(Δ*L1*), pBAD24-*clpY*(Δ*L2*), and pBAD24-*clpY*(Δ*L1*, Δ*L2*). The *E. coli* strain AC3112, a *lon clpQ clpY* triple mutant, was thereafter used as a host for cotransformation by pBAD33-*clpQ*$^+$ in combination with pBAD24-*clpY*$^+$or its derivatives. Since the two plasmids are compatible, the resultant cotransformant carrying pBAD24-*clpQ*$^+$ and pBAD33-*clpY*$^+$ was used as a positive control. Cells carrying both pBAD33 and pBAD24 plasmids were used as a negative control. As noted, AC3112 cells were sensitive to MMS at a low EOP ($\leqq 10^{-3}$) and with higher β-galactosidase activity of *cpsB*::*lacZ* due to induction of SulA and the stability of RcsA in the absence of both Lon and ClpYQ proteases. After arabinose induction, bacterial cells that coexpressed both ClpQ and ClpY (from pBAD33-*clpQ*$^+$ and pBAD24-*clpY*$^+$) showed decreased β-galactosidase activity (Fig. 4A, lane 2) and were MMS resistant (Fig. 4B, lane 2). Similar results were observed in bacteria that expressed ClpY(ΔL1) in the presence of ClpQ (Fig. 4A and B, lanes 3). Bacterial cells that expressed either ClpY(ΔL2), ClpY(ΔL1, ΔL2), or ClpY(ΔI+7Gly) in the presence of ClpQ showed higher β-galactosidase activity and increased MMS sensitivity compared to the negative control (Fig. 4A and B, compare lanes 4, 5, and 6 to lane 1). These results indicate that ClpY(ΔL1, ΔL2) and ClpY(ΔI+7Gly) molecules were not able to bind to their substrates for degradation by ClpQ. Since ClpY(ΔL1) is able to bind to SulA, the aa 175 to 209 loop in ClpY is apparently necessary for the delivery of substrates.

Western blotting of residual SulA and ClpQ, as well as ClpY and its derivative mutants. In vivo, residual SulA and ClpQ, as well as ClpY and its derivative mutants, were also detected in different bacterial strains using the Western blot assay. AC3112 cells carrying pBAD33-*clpQ*$^+$ with pBAD24-*clpY*$^+$ or its derivatives were transformed with pTH18kr-*ha-sulA*$^+$ and selected on LA medium supplemented with ampicillin, chloramphenicol, and kanamycin. Each bacterial cell was then grown on LB medium with the appropriate antibiotics plus 0.5% arabinose for the induction of ClpQ, as well as ClpY and its derivative mutants. After the bacterial cultures were grown to early log phase, IPTG (1 mM final concentration) was added to the medium; this led to the induction of HA-SulA. After 2 h of induction, the cell mixtures were diluted in series and plated for calculation of the EOP. Each bacterial strain had an EOP (the number of colonies on medium with IPTG induction divided by the number without IPTG induction) similar to that determined by the corresponding MMS test (data not shown). Protein samples were also extracted and subjected to Western blot analysis using anti-HA, anti-ClpQ, and anti-ClpY antibodies. The control strain with pTH18kr-*ha-sulA*$^+$, pBAD33, and pBAD24 was used for detection of HA-SulA (Fig. 5, top row, lane 1). Thus, residual SulA was observed in different bacterial strains expressing ClpY(ΔL2), ClpY(ΔL1, ΔL2), or ClpY(ΔI+7Gly) (Fig. 5, top row, lanes 4, 5, and 6). However, no SulA was observed in the bacterial strain that expressed wild-type ClpY or ClpY(ΔL1) in the presence of ClpQ (Fig. 5, top row, lanes 2 and 3). These results were consistent with the results of the MMS tests.

The induction of ClpQ was equally expressed in the bacterial strains, and the wild-type ClpY and its derivative mutants were mostly equally expressed, except for ClpY(ΔI+7Gly), which was expressed at a lower level (Fig. 5, top and middle rows, lanes 2 to 6).

A random selection of ClpY mutants map in the aa 175 to 209 loop region, and certain ClpY mutants are defective in the degradation of substrates in the presence of ClpQ. To identify the critical amino acids that affect SulA binding, a PCR randomized mutagenesis method was used to make a pool of mutations surrounding the I domain of ClpY. The DNA fragment that encompassed the entire I domain was first amplified by an error-prone PCR (see Materials and Methods). A PCR DNA fragment was amplified from the nucleotide corresponding to aa 63 and ended with the last nucleotide corresponding to aa 282. A genetic screen was then used in the yeast two-hybrid assays to select the color-changed colonies. The EGY48(p8op-*lacZ*) yeast, carrying pGilda-*sulA*$^+$, was transformed with a linear pB42AD-*clpY*$^+$ (with an NruI-linearized *clpY*$^+$ gene) mixed with a pool of the above-mentioned PCR-mutated DNA fragments. The PCR products were then homologously recombined with the linearized pB42AD-*clpY*$^+$ plasmids. The resulting transformants were subsequently

its derivatives with BD-SulA, and the AD-ClpY or its derivatives with BD-SulA(M89I). The error bars indicate standard deviations. (B) The yeasts were grown on *leu*-deficient media. The top row is the control, and the bottom row is AD-ClpY or various ClpY mutants with BD-SulA. (C) Western blot of ClpY and its derivatives in the AD fusion protein using an anti-HA antibody. (D) Assays of *cpsB*::*lacZ* expression and MMS tests for each bacterial condition. Each strain carries wild-type ClpY or the various ClpY mutants in the presence of ClpQ. At the top is the β-galactosidase level of the *cpsB*::*lacZ* expression of each bacterium. At the bottom is the EOP value for each bacterial condition in the MMS tests. (E) Western blots of ClpY, ClpQ, and HA-SulA for each bacterium. The top row is HA-SulA. The middle row is ClpQ and its derivative mutants. The bottom row is ClpY, as indicated.


A
β-galactosidase unit
10⁴
10³
10²
10¹
10⁰
pGilda
pGilda-sulA
pGilda-sulA(M891)
pB42AD
clpY
clpY(I186N)
clpY(E193L, E194L)
clpY(Q198L, Q200L)
B
pB42AD
clpY
clpY(I186N)
clpY(E193L, E194L)
clpY(Q198L, Q200L)
pGilda
pGilda-sulA
1 2 3 4 5
C
pB42AD
clpY
clpY(I186N)
clpY(E193L, E194L)
clpY(Q198L, Q200L)
64kDa
1 2 3 4 5
D
0.5% Ara
1% Glu
cpsB::lacZ units
250
200
150
100
50
0
pBAD24
clpY
clpY(I186N)
clpY(E193L, E194L)
clpY(Q198L, Q200L)
MMS EOP:
10-4
10-1
10-3
10-3
10-3
E
control
clpY
clpY(I186N)
clpY(E193L, E194L)
clpY(Q198L, Q200L)
SulA
ClpQ
ClpY
1 2 3 4 5

scored on X-Gal indicator plates. Dark-blue or white colonies were then selected. The pB42AD-*clpY*$^+$ derivatives bearing mutations in the *clpY*$^+$ structural gene were then extracted from the yeast cells, and the resulting plasmids were retransformed into EGY48(p8op-*lacZ*) cells carrying pGilda-*sulA*$^+$. The phenotypes of the newly transformed yeasts were repeatedly confirmed on both X-Gal medium and *leu*-deficient plates. The sites of mutations in the ClpY mutants were then identified by DNA sequencing. The mutations mapped mostly to an inner loop region (L2 loop), including M187I, A188S, L199Q, and N205K (Fig. 1). Compared to the positive control (yeast carrying AD-ClpY/BD-SulA), yeasts that expressed BD-ClpY (M187I) and AD-SulA were grown on *leu*-deficient medium and were dark blue on X-Gal medium, with higher β-galactosidase activities (Fig. 6A and B, compare lanes 3 to lanes 2). ClpY(L199Q), however, showed the lowest binding activity toward SulA; the yeast cells were white on the X-Gal plate and expressed lower β-galactosidase activity, and cell growth was barely observed on *leu*-deficient medium (Fig. 6A and B, lanes 5). Yeast expressing ClpY(A188S) or ClpY(N205K) with SulA grew slowly on *leu*-deficient plates and was light blue on X-Gal medium, with slightly reduced β-galactosidase activity (Fig. 6A and B, lanes 4 and 6). Similar phenotypes were observed when BD-SulA(M89I) was expressed in yeast (Fig. 6A, lanes 2 to 6). In addition, using the anti-HA antibody, AD-ClpY and its derivatives were observed in the yeast (Fig. 6C, lanes 2 to 6).

As indicated, the bacterial AC3112 cells that coexpressed ClpY(M187I), ClpY(A188S), or ClpY(N205K) with ClpQ under induction by arabinose showed MMS resistance and had decreased β-galactosidase activity of *cpsB*::*lacZ* (Fig. 6D, lanes 3, 4, and 6). However, the bacteria that expressed ClpY (L199Q) were MMS sensitive and had higher β-galactosidase activity (Fig. 6D, lane 5). Therefore, the natural substrates of these ClpY mutants, except for ClpY(L199Q), were able to be degraded.

The residual SulA and ClpQ, as well as ClpY and its derivatives, were then simultaneously detected in the above-mentioned bacterial strains. SulA accumulated in the bacterial cells that expressed ClpY(L199Q) (Fig. 6E, top row, lane 5). Little or no SulA accumulated in cells that expressed ClpY(M187I), ClpY(A188S), or ClpY(N205K) (Fig. 6E, top row, lanes 3, 4, and 6). ClpQ was expressed equally in each of the bacterial strains (Fig. 6E, middle row, lanes 2 to 6), and ClpY and its mutant derivatives were each equally expressed in all the bacterial strains (Fig. 6E, bottom row, lanes 2 to 6).

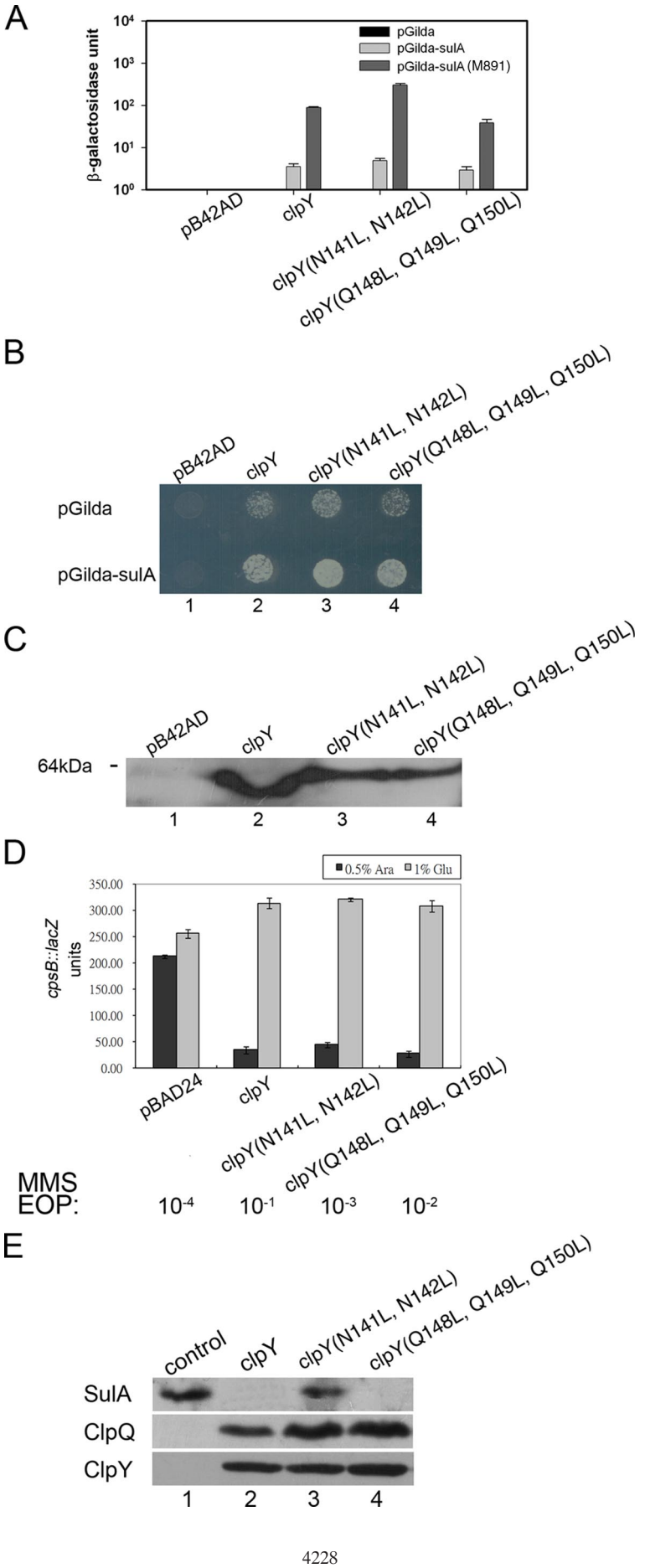
The site-directed ClpY(E193L, E194L) and ClpY(Q198L, Q200L) loop 2 mutants had enhanced moderate SulA-binding activity but no degradation by ClpQ. To further confirm that the hydrophobic side chains of aa 175 to 209 in ClpY take part in the binding of substrates, site-directed mutagenesis was used to construct single or double point mutations in the same region. These site-specific ClpY mutants had the substituted residues at a position(s) adjacent to those of the randomly selected residue(s). *clpY*(*I186N*), *clpY*(*E193L*, *E194L*), and *clpY*(*Q198L*, *Q200L*) were made in pB42AD plasmids subsequently designated pB42AD-*clpY*(*I186N*), pB42AD-*clpY* (*E193L*, *E194L*), and pB42AD-*clpY*(*Q198L*, *Q200L*). As a control, a pB42AD-*clpY*$^+$ (SmaI) plasmid carried the *clpY*$^+$ gene with a silent SmaI site (a unique SmaI site created by a silent mutation). These four plasmids were then individually transformed into the yeast EGY48(p8op-*lacZ*) carrying pGilda-*sulA*$^+$ or pGilda-*sulA*(*M89I*), and the resultant transformants were scored on X-Gal plates, had their β-galactosidase activities measured, and were tested on *leu*-deficient medium. ClpY(Q198L, Q200L) and ClpY(E193L, E194L) were significantly associated with SulA/SulA(M89I) (Fig. 7A and B, lanes 4 and 5), whereas ClpY(I186N) did not interact with SulA compared to the wild-type ClpY (Fig. 7A and B, compare lanes 3 to lanes 2). Thus, these results suggest that the hydrophobic groups of amino acids in the loop of ClpY are necessary for its binding activity toward the substrates. Also, AD-ClpY and its derivative mutants were expressed well in the yeast (Fig. 7C, lanes 2 to 5).

When AC3112 cells coexpressed ClpQ with ClpY(I186N), ClpY(E193L, E194L), or ClpYQ(Q198L, Q200L), each a bacterial strain showed MMS sensitivity and had higher β-galactosidase activity of *cpsB*::*lacZ* (Fig. 7D, lanes 3 to 5) than cells with the wild-type ClpY (SmaI) (Fig. 7D, lane 2, with lane 1 as a negative control). Therefore, these ClpY mutants showed defective activity for the degradation of the natural substrates.

The expression of ClpQ, as well as ClpY (SmaI) and its derivatives, from each bacterial strain is shown in Fig. 7E. Coincident with the MMS results, SulA accumulated in cells that expressed ClpY(I186N), ClpY(E193L, E194L), or ClpY (Q198L, Q200L) (Fig. 7E, top row, lanes 3 to 5), but not in cells that expressed the wild-type ClpY (SmaI) (Fig. 7E, top row, lane 2). It is likely that ClpY(I186N) is defective in substrate binding whereas ClpY(E193L, E194L) and ClpY (Q198L, Q200L) are defective in intracellular delivery of substrates for further degradation by ClpQ.

ClpY(N141L, N142L), a loop 1 mutant defective in complete degradation of SulA. In addition, to assess whether the residues in the L1 loop are involved in delivery activity, *clpY* (*N141L*, *N142L*) and *clpY*(*Q148L*, *Q149L*, *Q150L*), in which both *clpY* genes carry multiple mutations in the L1 loop, were made; the mutated genes were cloned into pB42AD; and the resultant plasmids were designated pB42AD-*clpY*(*N141L*, *N142L*) and pB42AD-*clpY*(*Q148L*, *Q149L*, *Q150L*), respectively. After transformation of pB42AD-*clpY*(*N141L*, *N142L*) and pB42AD-*clpY*(*Q148L*, *Q149L*, *Q150L*) with pGilda-*sulA*$^+$/*sulA*(*M89I*), all the cotransformants were scored on X-Gal plates and tested on *leu*-deficient medium. The yeast cells were

FIG. 7. Expression of *lacZ* and LEU2 in EGY48(p8op-*lacZ*) cells carrying different ClpY loop 2 mutants in the AD, as well as AD-ClpY (SmaI), for interaction with BD-SulA/SulA(M89I); the physiological activities for each bacterial condition are shown. (A and B) Yeasts with a pair of AD or BD fusion proteins, as indicated, were measured for β-galactosidase activity (A) or grown on *leu*-deficient medium (B). The error bars indicate standard deviations. (C) The ClpY mutants and wild-type ClpY (SmaI) AD fusion protein were detected in yeast using an anti-HA antibody. (D) *cpsB*::*lacZ* assays and MMS tests for each bacterial strain carrying the wild-type ClpY or its derivative mutants with ClpQ. (E) Western blots of HA-SulA, ClpQ, and ClpY and its derivatives for each bacterial strain.


A
β-galactosidase unit
pGilda
pGilda-sulA
pGilda-sulA (M891)
pB42AD
clpY
clpY(N141L, N142L)
clpY(Q148L, Q149L, Q150L)
B
pB42AD
clpY
clpY(N141L, N142L)
clpY(Q148L, Q149L, Q150L)
pGilda
pGilda-sulA
1
2
3
4
C
pB42AD
clpY
clpY(N141L, N142L)
clpY(Q148L, Q149L, Q150L)
64kDa
1
2
3
4
D
0.5% Ara
1% Glu
cpsB::lacZ units
pBAD24
clpY
clpY(N141L, N142L)
clpY(Q148L, Q149L, Q150L)
MMS EOP:
E
control
clpY
clpY(N141L, N142L)
clpY(Q148L, Q149L, Q150L)
SulA
ClpQ
ClpY
1
2
3
4

blue on the X-Gal medium, with higher β-galactosidase activity than the control, and grew on the *leu*-deficient medium (Fig. 8A and B, lanes 2, 3, and 4). As indicated, ClpY(N141L, N142L) and CpY(Q148L, Q149L, Q150L) significantly interacted with SulA/SulA(M89I) compared to the wild-type ClpY. Both ClpY(N141L, N142L) and ClpY(Q148L, Q149L, Q150L) were well expressed in yeast, as was the wild-type ClpY (Fig. 8C, compare lanes 3 and 4 to lane 2). The AC3112 bacterial cells that coexpressed ClpQ and ClpY(N141L, N142L) were MMS sensitive and had subtly increased β-galactosidase activity from *cpsB*::*lacZ* compared to the wild-type ClpY (Fig. 8D, compare lane 3 to lane 2). ClpY(Q148L, Q149L, Q150L), however, showed normal activity similar to that of the wild-type ClpY (Fig. 8E, compare lane 4 to lane 2). In the presence of ClpQ, SulA accumulated in AC3112 cells that expressed ClpY(N141L, N142L), but not in the cells that expressed ClpY (Q148L, Q149L, Q150L) (Fig. 8E, compare lanes 3 and 4 to lane 2). ClpQ and ClpY, with its derivatives, were expressed in all bacterial strains (Fig. 8E, lanes 2 to 4). Therefore, the ClpY (N141L, N142L) mutant appeared to be defective in substrate delivery for degradation by ClpQ.

DISCUSSION

One of the specific aims of this study was to determine whether domain I of ClpY is responsible for the recognition of its natural substrates. Using a ClpY(ΔI+7Gly) mutant that lacked the I domain but contained seven Gly residues as a hinge between the N and C domains, we showed that domain I is required for association with SulA in the yeast two-hybrid assays. In addition, ClpY and ClpY(ΔI+7Gly) mutants both interacted with ClpQ(E61C). Thus, the protruding domain I of ClpY functions in cellular target recognition. However, neither ClpYX, with a C domain substituted from ClpX, nor a ClpY (ΔC) mutant, completely lacking the C domain, associated with ClpQ(E61C). These data suggest that the C domain of ClpY is necessary for association and dissociation of substrates with ClpQ.

It was demonstrated in vitro that a region (aa 175 to 209) in the I domain of ClpY is required for the degradation of MBP-SulA by ClpQ (33). Residues 175 to 209 constitute an α loop in the X-ray structure of the analogous ClpY of *H. influenzae* (34). Here, a ClpY(ΔL2) mutant, lacking aa 175 to 209, was shown to associate with SulA. Accordingly, ClpY(ΔL1), with a deletion of aa 137 to 150, had slightly less proteolytic activity for MBP-SulA (33). ClpY(ΔL1, ΔL2) did not associate with SulA, and in the presence of ClpQ, the mutant complex led to no degradation of SulA or RcsA. However, ClpY(ΔL1) and ClpY(ΔL2), lacking either loop, were still able to associate with SulA; this observation suggests that both loops in ClpY are able to bind to its natural substrates. In addition, since loop 2 (L2) in ClpY was shown to have much stronger binding to SulA, and ClpY(ΔL2) could not degrade SulA in the presence of ClpQ, it is likely that this loop (aa 175 to 209) in ClpY plays a role in the fastening and delivery of natural substrates.

Moreover, in randomly selected ClpY mutants with altered SulA-binding affinities, the mutations mapped within the aa 175 to 209 loop region. Interestingly, the substituted amino acids, such as ClpY(A188S), ClpY(I186N), ClpY(L199Q), and ClpY(N205K), with polar or more positively charged side groups led to less SulA-binding activity. However, in ClpY (L199Q) and ClpY(I186N), both molecules were expressed at lower levels in the yeasts. Our previous results showed that AD-ClpY was not so easily detected if no interactive protein was present (19). This observation also supports the notion that ClpY(L199Q) showed the least binding activity and ClpY(I186N) displayed no binding to SulA. Conversely, hydrophobic groups in the side chains of the substituted residues, e.g., ClpY(M187I), led to enhanced SulA binding. Thus, the hydrophobic side chains of the residues in the aa 175 to 209 loop are likely involved in an intermodular association between the chaperone and its substrates.

Both ClpY(E193L, E194L) and ClpY(Q198L, Q200L) mutants were shown to associate with SulA but were unable to degrade it in the presence of ClpQ. Therefore, both molecules were apparently unable to deliver their natural substrates for degradation by ClpQ, and as such, neither of the ClpY mutants could deliver the substrates to the central pore of the cylindrical complex. In contrast, ClpY(M187I) showed higher SulA-binding activity, and the substrates were degraded in the presence of ClpQ. Moreover, ClpY(A188S) and ClpY(N205K) showed slightly less binding to SulA, and only the ClpY (N205K) mutant showed a minimal decrease in substrate degradation in the presence of ClpQ. Therefore, the ClpY (M187I), ClpY(A188S), and ClpY(N205K) mutants maintained their abilities to deliver substrates for degradation by ClpQ. Interestingly, a small amount of residual SulA was routinely detected when ClpY(N205K) and ClpQ were expressed in the protease complex, suggesting that the substrates were being progressively degraded. Additionally, the loop 1 mutant, ClpY(Q148L, Q149L, Q150L), binds to SulA, but it does not efficiently degrade the protein. Also, with this mutant protein, in AC3112 cells that had ClpQ, there was always slightly increased β-galactosidase activity of *cpsB*::*lacZ* compared to the cells that carried the wild-type ClpY. These results indicate that loop 1 is most probably also involved in substrate delivery. Notably, the binding and delivery activities of ClpY demonstrated here likely occurred prior to the unfolding/translocation of substrates via the pore site into the inner core for degradation by ClpQ.

Using a phage P22 Arc artificial substrate, it was previously reported that ClpY recognizes and binds to the fluorescence-labeled Arc in the presence of ATPγS and Mg^{2+} (3). The

FIG. 8. Expression of *lacZ* and LEU2 in EGY48(p8op-*lacZ*) cells carrying different ClpY loop 1 mutants in the AD, as well as AD-ClpY (SmaI), for interaction with BD-SulA/SulA(M89I); physiological activities for each bacterial condition. (A and B) Yeasts were measured for β-galactosidase activity with each pair of AD and BD fusion proteins as indicated (A) or grown on *leu*-deficient medium (B). The error bars indicate standard deviations. (C) Western blotting of AD-ClpY (SmaI) and various AD-ClpY mutants in yeasts using an anti-HA antibody. (D) *cpsB*::*lacZ* assays and MMS tests for each bacterial strain carrying wild-type ClpY or its derivative mutants with ClpQ. (E) Western blots of HA-SulA and ClpY and its derivatives, as well as ClpQ, in the bacterial strains.

nucleotide-bound ATP-ClpY molecules are responsible for the recognition of substrates in the ClpYQ complex. It was also later shown that three or four ATPs were required for the ClpYQ complex in the process of recognition, unfolding/translocation, and degradation of the substrate (39). In this study, we demonstrated that the double loops aa 137 to 150 and aa 175 to 209 in domain I of ClpY are essential for association of the natural substrates and that the aa 175 to 209 loop is likely involved in gripping the substrates. However, this loop is not the sole determinant of the binding affinity of ClpYQ for its substrate, since different binding constants for the ClpYQ complex were determined when it was conjugated to the various Arc fragments (3). Also, ClpY(ΔL1) and ClpY(ΔL2) showed enhanced binding to SulA(M89I), suggesting that other binding sites in domain I in the above-mentioned mutants could be involved in substrate tethering. Accordingly, we identified a pair of ClpY mutants containing multiple mutations outside the double-loop region in domain I, and with both molecules, SulA binding was affected (data not shown).

Based on our results, we conclude that the external loop (aa 137 to 150) and the inner loop (aa 175 to 209) function in cellular substrate binding and that the inner loop is responsible for the gripping of natural substrates, such as SulA, and possibly for intracylindrical delivery. However, the outer loop (aa 137 to 150) may also have an ancillary function in substrate delivery. We suggest that the inner loop grips the substrates, supporting a model in which both loops are active in the generation of ATP-dependent conformational changes that channel substrates into the inner chamber of the ClpYQ cylinder. Since a conformational change of the α/β domain (N) in ClpY occurs when ATP is consumed, our observations are consistent with a similar mechanism of action for the protruding domain I that may likely drive substrate delivery (36).

This mechanism is consistent with the observation that ClpY acts as a robust unfoldase (3) and that adaptive configurations may allow the efficient unfolding of natural substrates. Further studies are needed to examine the interplay between the loops of domain I and the pore sites in ClpY. Specific attention should be paid to the binding, delivery, unfolding, and translocation of substrates into the inner core of ClpQ and the coupling of these activities with the activities of bound ATP and ATPase activities.

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REFERENCES

1. **Azim, M. K., W. Goehring, H. K. Song, R. Ramachandran, M. Bochtler, and P. Goettig.** 2005. Characterization of the HslU chaperone affinity for HslV protease. Protein Sci. **14:**1357–1362.
2. **Bochtler, M., C. Hartmann, H. K. Song, G. P. Bourenkov, H. D. Bartunik, and R. Huber.** 2000. The structures of HsIU and the ATP-dependent protease HsIU-HsIV. Nature **403:**800–805.
3. **Burton, R. E., T. A. Baker, and R. T. Sauer.** 2005. Nucleotide-dependent substrate recognition by the AAA^+ HslUV protease. Nat. Struct. Mol. Biol. **12:**245–251.
4. **Chuang, S. E., V. Burland, G. Plunkett III, D. L. Daniels, and F. R. Blattner.** 1993. Sequence analysis of four new heat-shock genes constituting the *hslTS*/*ibpAB* and *hslVU* operons in *Escherichia coli*. Gene **134:**1–6.
5. **Gottesman, S.** 2003. Proteolysis in bacterial regulatory circuits. Annu. Rev. Cell Dev. Biol. **19:**565–587.
6. **Guzman, L.-M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
7. **Haney, S. A., E. Glasfeld, C. Hale, D. Keeney, Z. He, and P. de Boer.** 2001. Genetic analysis of the *Escherichia coli* FtsZ-ZipA interaction in the yeast two-hybrid system. Characterization of FtsZ residues essential for the interactions with ZipA and with FtsA. J. Biol. Chem. **276:**11980–11987.
8. **Hashimoto-Gotoh, T., M. Yamaguchi, K. Yasojima, A. Tsujimura, Y. Wakabayashi, and Y. Watanabe.** 2000. A set of temperature sensitive-replication/-segregation and temperature resistant plasmid vectors with different copy numbers and in an isogenic background (chloramphenicol, kanamycin, *lacZ*, *repA*, *par*, *polA*). Gene **241:**185–191.
9. **Ishikawa, T., M. R. Maurizi, D. Belnap, and A. C. Steven.** 2000. Docking of components in a bacterial complex. Nature **408:**667–668.
10. **Kanemori, M., K. Nishihara, H. Yanagi, and T. Yura.** 1997. Synergistic roles of HslVU and other ATP-dependent proteases in controlling in vivo turnover of σ^{32} and abnormal proteins in *Escherichia coli*. J. Bacteriol. **179:**7219–7225.
11. **Kanemori, M., H. Yanagi, and T. Yura.** 1999. Marked instability of the σ^{32} heat shock transcription factor at high temperature. Implications for heat shock regulation. J. Biol. Chem. **274:**22002–22007.
12. **Kessel, M., W. F. Wu, S. Gottesman, E. Kocsis, A. C. Steven, and M. R. Maurizi.** 1996. Six-fold rotational symmetry of ClpQ, the *E. coli* homolog of the 20S proteasome, and its ATP-dependent activator, ClpY. FEBS Lett. **398:**274–278.
13. **Khattar, M. M.** 1997. Overexpression of the *hslVU* operon suppresses SOS-mediated inhibition of cell division in *Escherichia coli*. FEBS Lett. **414:**402–404.
14. **Kim, D. Y., and K. K. Kim.** 2003. Crystal structure of ClpX molecular chaperone from *Helicobacter pylori*. J. Biol. Chem. **278:**50664–50670.
15. **Kuo, M.-S., K.-P. Chen, and W. F. Wu.** 2004. Regulation of RcsA by the ClpYQ (HslUV) protease in *Escherichia coli*. Microbiology **150:**437–446.
16. **Kwon, A. R., B. M. Kessler, H. S. Overkleeft, and D. B. McKay.** 2003. Structure and reactivity of an asymmetric complex between HslV and I-domain deleted HslU, a prokaryotic homolog of the eukaryotic proteasome. J. Mol. Biol. **330:**185–195.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
18. **Lau-Wong, I. C., T. Locke, M. J. Ellison, T. L. Raivio, and L. S. Frost.** 2008. Activation of the Cpx regulon destabilizes the F plasmid transfer activator, TraJ, via the HslVU protease in *Escherichia coli*. Mol. Microbiol. **67:**516–527.
19. **Lee, Y.-Y., C.-F. Chang, C.-L. Kuo, M.-C. Chen, C. H. Yu, P.-I. Lin, and W. F. Wu.** 2003. Subunit oligomerization and substrate recognition of the *Escherichia coli* ClpYQ (HslUV) protease implicated by in vivo protein-protein interactions in the yeast two-hybrid system. J. Bacteriol. **185:**2393–2401.
20. **Lien, H.-Y., C.-H. Yu, C.-M. Liou, and W. F. Wu.** 2009. Regulation of $clpQ^+Y^+$ ($hslV^+U^+$) gene expression in *Escherichia coli*. Open Microbiol. J. **3:**29–39.
21. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
22. **Missiakas, D., F. Schwager, J. M. Betton, C. Georgopoulos, and S. Raina.** 1996. Identification and characterization of HsIV HsIU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in *Escherichia coli*. EMBO J. **15:**6899–6909.
23. **Munavar, H., Y. Zhou, and S. Gottesman.** 2005. Analysis of the *Escherichia coli* Alp phenotype: heat shock induction in *ssrA* mutants. J. Bacteriol. **187:**4739–4751.
24. **Nishii, W., and K. Takahashi.** 2003. Determination of the cleavage sites in SulA, a cell division inhibitor, by the ATP-dependent HslVU protease from *Escherichia coli*. FEBS Lett. **553:**351–354.
25. **Park, E., J. W. Lee, S. H. Eom, J. H. Seol, and C. H. Chung.** 2008. Binding of MG132 or deletion of the Thr active sites in HslV subunits increases the affinity of HslV protease for HslU ATPase and makes this interaction nucleotide-independent. J. Biol. Chem. **283:**33258–33266.
26. **Park, E., Y. M. Rho, O. J. Koh, S. W. Ahn, I. S. Seong, J. J. Song, O. Bang, J. H. Seol, J. Wang, S. H. Eom, and C. H. Chung.** 2005. Role of the GYVG pore motif of HslU ATPase in protein unfolding and translocation for degradation by HslV peptidase. J. Biol. Chem. **280:**22892–22898.
27. **Ramachandran, R., C. Hartmann, H. K. Song, R. Huber, and M. Bochtler.** 2002. Functional interactions of HslV (ClpQ) with the ATPase HslU (ClpY). Proc. Natl. Acad. Sci. USA **99:**7396–7401.
28. **Rohrwild, M., O. Coux, H. C. Huang, R. P. Moerschell, S. J. Yoo, J. H. Seol, C. H. Chung, and A. L. Goldberg.** 1996. HslV-HslU: a novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. Proc. Natl. Acad. Sci. USA **93:**5808–5813.
29. **Rohrwild, M., G. Pfeifer, U. Santarius, S. A. Muller, H. C. Huang, A. Engel, W. Baumeister, and A. L. Goldberg.** 1997. The ATP-dependent HslVU protease from *Escherichia coli* is a four-ring structure resembling the proteasome. Nat. Struct. Biol. **4:**133–139.

30. **Seong, I. S., M. S. Kang, M. K. Choi, J. W. Lee, O. J. Koh, J. Wang, S. H. Eom, and C. H. Chung.** 2002. The C-terminal tails of HslU ATPase act as a molecular switch for activation of HslV peptidase. J. Biol. Chem. **277:**25976–25982.
31. **Seong, I. S., J. Y. Oh, S. J. Yoo, J. H. Seol, and C. H. Chung.** 1999. ATP-dependent degradation of SulA, a cell division inhibitor, by the HslVU protease in *Escherichia coli*. FEBS Lett. **456:**211–214.
32. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. **Song, H. K., C. Hartmann, R. Ramachandran, M. Bochtler, R. Behrendt, L. Moroder, and R. Huber.** 2000. Mutational studies on HslU and its docking mode with HslV. Proc. Natl. Acad. Sci. USA **97:**14103–14108.
34. **Sousa, M. C., C. B. Trame, H. Tsuruta, S. M. Wilbanks, V. S. Reddy, and D. B. McKay.** 2000. Crystal and solution structures of an HslUV protease-chaperone complex. Cell **103:**633–643.
35. **Wang, J., J. J. Song, M. C. Franklin, S. Kamtekar, Y. J. Im, S. H. Rho, I. S. Seong, C. S. Lee, C. H. Chung, and S. H. Eom.** 2001. Crystal structures of the HslVU peptidase-ATPase complex reveal an ATP-dependent proteolysis mechanism. Structure **9:**177–184.
36. **Wang, J., J. J. Song, I. S. Seong, M. C. Franklin, S. Kamtekar, S. H. Eom, and C. H. Chung.** 2001. Nucleotide-dependent conformational changes in a protease-associated ATPase HsIU. Structure **9:**1107–1116.
37. **Wu, W.-F., Y. N. Zhou, and S. Gottesman.** 1999. Redundant in vivo proteolytic activities of *Escherichia coli* Lon and the ClpYQ (HslUV) protease. J. Bacteriol. **181:**3681–3687.
38. **Wu, W. F., C.-S. Yang, H.-Y. Lien, F.-C. Hsieh, and C.-Y. Chang.** 2008. HslUV (ClpYQ), an ATP-dependent protease with unique characteristics, p. 115–143. *In* E. Kutejova (ed.), ATP-dependent proteases. Trivandrum, Kerala, India.
39. **Yakamavich, J. A., T. A. Baker, and R. T. Sauer.** 2008. Asymmetric nucleotide transactions of the HslUV protease. J. Mol. Biol. **380:**946–957.
40. **Zolkiewski, M.** 2006. A camel passes through the eye of a needle: protein unfolding activity of Clp ATPases. Mol. Microbiol. **61:**1094–1100.