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

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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 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 the 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, which lie in the 137-150 aa (loop 1) and 175-209 aa (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 the random PCR mutagenesis allowed the isolation of ClpY mutants that displayed a range of binding activity with SulA, including a mutant with no SulA-binding at all. Subsequently, via methyl methanesulfonate (MMS) tests and *cpsB::lacZ* assays, e.g. SulA and RcsA as targets, we concluded that the 175-209 aa of loop 2 is involved in the gripping of natural substrates, and it is likely that both loops, the 137-150 aa and 175-209 aa, of the ClpY domain I may assist in the delivery of substrates into the inner core for ultimate degradation by ClpQ.
**INTRODUCTION**

*E. 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 constitute 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, 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 demonstrated that Cpx-mediated TraJ degradation by ClpYQ was stress-dependent (18). Additionally, the basal and heat shock-induction of the *clpQ*+Y+ operon was mediated solely by the RpoH factor (4, 20), and the stem-loop structure in the 5’ untranslabeled region (5’UTR) of *clpQ*+Y+ mRNA(s) was shown necessary for the stability of its transcript(s) (20).

The ClpY molecule is divided into three domains: the N-terminal domain (N; residues 2-109/244-332), I-intermediate domain (I; residues 110-243) and C-terminal (C; residues 333-443) domain (2) (Fig. 1). The N domain has an ATPase activity (in 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 association of ClpY with ClpQ observed suggests not necessarily for translocation of the substrate through the rather small central core of the HslUV hexamer; moreover ClpY might deliver substrates through altered active state of an affinity for ClpQ (2, 27, 33). By contrast, based on the electron microscopy (EM) images, in the docking mode, domain I appears to protrude from the ClpYQ complex with the process of substrate delivery unresolved (in Fig. 1) (9, 29, 36). To investigate the above discrepancy, a deletion mutant ClpYΔ(175~209 aa) 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 is capable of stimulating ClpQ proteolytic activity (27, 30), and the G90Y91V92G93 pore motif of ClpY is involved in the unfolding and translocation of protein substrates into the inner core for the 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 (SPR), it has been demonstrated that ClpY increases 5-fold in binding affinity towards ClpQ while it is associated with MBP-SulA (1). However, a deletion of the Thr active site in ClpQ, also increases 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 requisite for substrate recognition (3). Additionally, asymmetric binding and hydrolysis of ATP(s) were demonstrated \textit{in vitro} for degradation of the substrates in the ClpYQ complex (39). The ClpYQ protease, for its physiological role and biochemical function in the eubacteria or in the higher organisms, was recently reviewed in Wu \textit{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 recognition of its natural substrates while domain C is necessary for association with ClpQ. The loop 175-209 aa plays a role in substrate fastening. We further identified two specific loops (137-150 aa and 175-209 aa) that together in domain I of ClpY are required for association with its natural substrates. In addition, our results suggest that both loops, 137-150 aa and 175-209 aa, of ClpY are likely also involved in substrate delivery for degradation by ClpQ.
MATERIALS AND METHODS

Strains and plasmids

The yeast *Saccharomyces cerevisiae* EGY48 (MATa, his3, trp1, ura2, *lexA*<sub>op(x6)</sub>-LEU2) reporter strain, which carries *p8op-lacZ* plasmids and *lexA*<sub>op(x6)</sub>-LEU2 integrated into the chromosome, was obtained from Clontech (Palo Alto, CA, USA). *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. The *E. coli* AC3112 (lon, ΔclpQY, cpsB::lacZ) (15) was used as a host for the MMS test and β-galactosidase assay for *cpsB::lacZ* expression. The plasmids pB42AD-<i>clpY</i><sup>+</sup>, pB42AD-<i>sulA</i><sup>+</sup>, pB42AD-<i>sulA</i><sup>M89I</sup>, pGilda-<i>sulA</i><sup>+</sup> and pGilda-<i>sulA</i><sup>M89I</sup> 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 broth (LB). For yeast growth, yeast peptone dextrose (YPD) and synthetic drop-out (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 (20). Additional supplements were added at the following final concentrations: glucose (Glc) 2%, galactose (Gal) 2%, raffinose (Raf) 1%, ampicillin (Ap) 100 µg/ml, kanamycin (Kn) 25 µg/ml, chloramphenicol (Cm) 34 µg/ml, X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) 80 µg/ml.

PCR randomized mutagenesis in domain I locus and the selection of ClpY mutants for an altered SulA binding in yeast two-hybrid assays

Mutations in DNA fragments of the ClpY domain I were generated by an error-prone polymerase chain reaction (PCR) amplification of the target region from pB42AD-<i>clpY</i><sup>+</sup> using power *Tag* DNA polymerase (German) in reaction conditions that favored incorporation of mutations (i.e. using Mn<sup>2+</sup> instead of Mg<sup>2+</sup>). Oligonucleotide primers, for PCR randomized amplification of domain I, were: forward primer 5’-AAAACCTGAAATCGCCCGTCG-3’ and reverse primer 5’-CAGCAGGTCACGCTGAACGC-3’. The two primers were annealed to the
cplY+-plasmid template for amplification of DNA sequences from the nucleotides of the 63th aa to the end of the 282th aa of ClpY. PCR products that encompassed the region of domain I were therefore made for a homologous recombination when co-transformed with linearized pB42AD-cplY+ (cut with NruI in the cplY+ gene) into EGY48 carrying pGilda-sulA+. This method has been described previously (7). Recombinants were then selected by growing the yeast on SD-Trp-His-Ura. The interaction between ClpY mutants and SulA was tested by scoring the resulting transformants of each on the X-gal minimal media with an addition of raffinose and galactose. White or dark blue colonies were saved for the further isolation of plasmids. The extracted plasmids were subsequently transformed into KC-8, and the resultant transformants were selected for Trp+ on GM medium plus DO without an addition of tryptophan. Plasmid pB42AD carrying cplY+ and its mutant derivatives were recovered from Trp+ colonies. Plasmids that expressed full-length cplY+ and its derivatives were verified again after retransformation into EGY48 [p8op-lacZ] carrying pGilda-sulA+ by scoring their newly transformants on an X-gal medium and testing on a Leu-depleted plate. The isolated plasmids, with no auto-activation of the reporters in the yeast, were then subjected to the DNA sequencing analysis to determine the site of the mutation(s).

To assess the expression of the reporters, the yeast strain EGY48 [p8op-lacZ] co-expressing BD- and AD- hybrid proteins was then subjected to the β-galactosidase analysis and was scored on the X-gal plate in addition to the leu+ selective assays using the methods as described earlier (19). Each assay was performed at least three times and at each time, the β-galactosidase analysis was assayed in triplicate for the yeast cells.

Construction of plasmids and the 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. (1984) (32) or by using cplY+, cplQ+ plasmids or their derivatives as the templates as listed before (19). However, plasmid pGilda-clpYX was constructed using two-step PCR amplification. The forward primer (F1) 5’CGCGGATCCATATGTCAGGGAACCCCC’ with the BamHI cutting site (underlined) and 3’-reverse primer (R1) 5’ATCAGGCTTCTTCGCTCCAGTCAACGCGGATTGG3’ were used for an amplification of a portion of cplY+ gene encoding the first amino acid to the 333th residue. The other primer sets, (F2) 5’CCAATCCGTTGAAACTGGGAGCGAAG
AAGCTCTGAT3’ and (R2) 5’CCGCCGCTCGAGTTATTCACCAGATGCCTG3’ with XhoI site (underlined), were used to amplify part of the clpX gene encoding the C-terminal domain from the 319th to the 424th amino acid residues. After Gel-M purification, these two PCR products were then taken as a 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’CCCCGCTCGAGGATGCTCTGTGAAATGACCCCA3’ (F3) with an XhoI site 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-clpYΔL1 (Δ137-150 aa), -clpYΔL2 (Δ175-209 aa), -clpYΔ1.ΔL2 (Δ137-150 aa and Δ175-209 aa) and -clpYΔI+7Gly [with domain I deleted but with 7 Gly(s) insertion] were constructed by two-step PCR methods. We used primer F4: 5’CCG GAA TTC ATGTCTGAAATGACCCCA-3’ (EcoRI site is underlined) and R3: 5’CTTTTTTGTGTCTCGATTCTTTTGTC-3’ for the front DNA fragment of clpYΔL2, and F5:5’AGAAATCGAGAAGCAAAAGCGCGTAAG3’ and R4: 5’CCCGC GGA TCC TTA TAG GAT AAA ACG GCT C3’ (BamHI site is underlined) for the rest of the DNA fragment for clpYΔL2. The resulting two PCR fragments were then used as a template for F3 and R4 amplification. The resulting DNA fragment encoding ClpYΔL2 was cloned into pGilda at EcoRI and BamHI sites. A similar two-step PCR method was used to construct plasmids clpYΔL1 and clpYΔ1.ΔL2. For clpYΔL1, we used the F3 primer and the R5 reverse primer 5’GA CGG TTC GAT CAG CAC GTC GAG AAT AC3’ for the front portion as well as the F6 primer 5’ C GTG CTG ATC GAA CCG TCC GCT GCT CGT CAG3’ and R4 for the remaining portion. For clpYΔ1.ΔL2, we used the internal primer: R6: 5’ CC ACC GCC ACC GCC ACC GCC TTT CAC GCC GCC GCC ATCG3’ and F3 primer for the front portion, and F7: 5’GC GTG GCC GTG GCC GTG GCC ATC GAC GCT GTT GAG CAG3’ and R4 primer for the remaining portion. Plasmid pGilda-clpYΔL1.ΔL2 was constructed using pGilda-clpYΔL2 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 a SmaI site to the middle of the clpY+ gene [CCT CCG GCC→CCC CCG GCC [(189th, 190th, 191th aa)] with no alterations in the encoded residues. This was accomplished by cloning the front portion of the clpY gene
encoding aa 1-190 (with EcoRI-SmaI at ends) and the rest of the gene encoding aa 191-443 (with SmaI-BamHI 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\(^{E193L,E194L}\), clpY\(^{Q198L,Q200L}\) are the derivatives of clpY\(^{+}\) (SmaI). Plasmids pB42AD-clpY\(^{N141L,N142L}\) and clpY\(^{Q148L,Q149L,Q150L}\) were constructed again by a 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 the DNA sequencing and were shown in Fig. 1. The other plasmid, pGilda-clpY\(^{+}\), was used as described in a previous study (19). The plasmids pB42AD-clpY\(^{+}\), pB42AD-clpY\(^{+}\), pB42AD-clpY\(^{+}\) and pB42AD-clpY\(^{Q7Gly}\) were constructed by cutting the 1.2 Kb DNA fragments out of the four corresponding plasmids separately, and all these four fragments were individually ligated into pB42AD at the EcoRI-XhoI sites, and the procedures for cloning were as described above.

The PCR amplification of the clpQ\(^{+}\) gene, including the upstream 63 bps untranslated leader sequences, was performed using the ClpQ-N terminal primer

\[5'\text{GAATTCCAGCTCGGTACC}GCATTATGCCCCGTA3'\]

and ClpQ-C terminal primer

\[5'\text{GGGGCTGCAGTTACGCTTTGTAGCT}3'\]. 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 site of pBAD24-NdeI (a construct that generates a new NdeI site in 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.

The MMS test and \(\beta\)-galactosidase assays for cpsB::lacZ expression

The E. coli AC3112 cells (lon\(^{-}\), \(\Delta\text{clpQY, cpsB::lacZ}\)) (15) carrying the plasmid pBAD33-clpQ\(^{+}\) in combination with pBAD24-clpY\(^{+}\) or with mutant clpY-relevant plasmids were grown overnight on LB agar with an ampicillin (50 \(\mu\)g/ml) and chloramphenicol (12.5 \(\mu\)g/ml) as well as glucose (1 %) or arabinose (0.5 %). One percent of the amount of an overnight culture was
inoculated into fresh media with the identical ingredients, and the cell culture was grown to log phase. 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 an addition of glucose (1 %) or arabinose (0.5 %). Efficiency of plating was calculated by comparing the titers of colonies on LA media with added MMS and arabinose divided by those of colonies on LA plus arabinose. Half of the log-phase cells were subjected to the β-galactosidase assay in triplicate using Miller units (21).

Construction of ha-sulA⁺ 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 ha-tag in front of sulA⁺. The forward primer: 5' GTACC CAT ATG TAC CCT TAT GAT GTG CCA GAT TAT3', and the reverse primer: 5' GGGGATCCGGATCC TTA ATG ATA TAC AAA TTA GAG TG3' 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 an lacIQ marker.

AC3112 variants carrying pBAD33-clpQ⁺ (chl²) with a combination of pBAD24-clpY⁺ (amp³), or its derivative plasmids were each transformed with pTH18kr-ha-sulA⁺ (kan⁶), 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 media with 0.5 % arabinose and the appropriate antibiotics. Dilutions (1:100) of the overnight bacterial cultures were inoculated into the fresh media and were grown with shaking at 30 °C. After three hours of growth to OD₆₀₀ = 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 OD₆₀₀ was recorded. Cellular extracts were then collected and separated on 12.5 % SDS-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. Western blots were developed using
enhanced chemiluminescence (Pierce).
RESULTS

Based on the X-ray crystal structures (2) and EM analysis (9), as well as the investigation of
H. influenza (35), questions were raised about whether the E. coli ClpYQ complex has the I
domain of ClpY oriented towards or away from the ClpQ hexamer. Up to now, we demonstrated
that ClpY (fused with an AD-domain) interacts with SulA (fused with a BD-domain) in the yeast-
two hybrid assays (19). We showed that an artificial transcriptional factor was reconstituted,
when the yeast EGY48[p8op-lacZ] expresses both AD-ClpY and BD-SulA. When both ClpY
and SulA were associated with each other under an induction by an addition of two sugars,
raffinose and galactose, the lacZ and leu2 genes were activated. Yeast transformants were
therefore grown on leu+ selective media (SD media, plus two sugars, without an addition of the
leucine) and were light blue on X-gal media. 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.

No interaction of ClpYΔI+7Gly and ClpYΔIΔL1ΔL2 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 clpYΔI+7Gly gene, with the domain I gene deleted in clpY but with 7 glycine codons
inserted at a site between domains N and C. This deletion mutant has a similar configuration as
ClpY on an analysis of its X-ray crystal structure (16). This DNA fragment was cloned into the
pGilda vector, and the resulting plasmid was designated pGilda-clpYΔI+7Gly. This plasmid was
thereafter transformed into the yeast EGY48[p8op-lacZ] carrying pB42AD-sulA+, and the
resultant transformants were selected on SD media lacking uracil, tryptophan, and histidine (SD-
U-W-H). The yeasts carrying BD-ClpYΔIΔL+/AD-SulA were white on X-gal plates with a lower
β-galactosidase activity and did not grow on leu-deficient media (Fig. 2A, 2C and 2D, lane 3);
these results were similar to those of the negative control (Fig. 2A, 2C and 2D lane 1, cells
carrying pGilda and pB42AD-sulA+). Therefore, no interaction was observed between
ClpYΔIΔL+/7Gly and SulA. In contrast, yeasts with BD-ClpY/AD-SulA or BD-SulA/AD-ClpY, as a
separately positive control, were light blue and had a subtle increase in β-galactosidase activity
and appeared Leu+ on the selective media (Fig. 2A, 2C and 2D, lane 2 and Fig. 2B, lane 2; as
compared to the lane 1). When SulA$^{M89I}$ was instead expressed in yeasts, it exhibited enhanced interaction with ClpY, i.e. the 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 of the yeast strains in which SulA was expressed (Fig. 2A, 2C and 2D, lane 2 to 6).

Accordingly, the ClpY$^{Δ(137-150 \text{ aa})}$ mutant, carrying a deletion of 137~150 aa, maintained the most activity for degradation of MBP-SulA in the presence of ClpQ (33). In contrast, a ClpY$^{Δ(175-209 \text{ aa})}$ mutant, with a deletion of 175~209 aa, apparently lost its activity to degrade the substrate by ClpQ (33). The reason for this observation remains unclear. Thus, we investigated whether these double loops in domain I of ClpY have the functional activity regarding to the recognition of the substrates. Two mutants with a single loop deletion, $clpY^{Δ(137-150 \text{ aa})}$ and $clpY^{Δ(175-209 \text{ aa})}$, were made in series and then were individually cloned into pGilda, and the resulting plasmids designated pGilda-$clpY^{ΔL1}$ and pGilda-$clpY^{ΔL2}$, respectively. A double loop deletion, $clpY^{Δ(137-150 \text{ aa}), Δ(175-209 \text{ aa})}$, was made as well and cloned into pGilda and the resulting plasmid was designated pGilda-$clpY^{ΔL1, ΔL2}$. Each plasmid was co-transformed with pB42AD-sulA$^+$ into the yeast EGY48[p8op-lacZ] and the resultant co-transformants, carrying the plasmid pair, were scored on the X-gal plates and tested on leu-deficient media. Yeast carrying ClpY$^{ΔL1, ΔL2}$/SulA were white on X-gal plates with lower $β$-galactosidase activity and also did not grow on leu-deficient media (Fig. 2A, 2C and 2D, lane 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, 2C and 2D, lane 4 and 5). The yeast colonies carrying BD-ClpY$^{ΔL1}$/AD-SulA (SulA$^{M89I}$) had much higher $β$-galactosidase activity than that of the yeast strains carrying BD-ClpY$^{ΔL1, ΔL2}$/AD-SulA (SulA$^{M89I}$) or BD-ClpY$^{ΔL2}$/AD-SulA (SulA$^{M89I}$) (Fig. 2A and 2C, lane 4, 5 and 6). Therefore, the 175~209 aa of ClpY is likely involved in the fastening or initial binding of the SulA molecule.

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$^{ΔL_1}$, ClpY$^{ΔL_2}$, ClpY$^{ΔL_1, ΔL_2}$ and ClpY$^{ΔL_1, ΔL_2, +7 Gly}$ associated with ClpQ$^{E61C}$ but not ClpY$^{ΔC}$ and ClpYX

We also tested interaction of the wild-type ClpY and the above ClpY derivative mutants with ClpQ$^{E61C}$. Accordingly, E61-ClpQ is bridged with R440- ClpY in the X-ray crystal structure (29). The ClpQ$^{E61C}$ not only has a higher affinity towards ClpY; also it retains normal proteolytic activity (our unpublished data). The pGilda-clpY*, -clpY$^{ΔL_1}$, -clpY$^{ΔL_2}$, -clpY$^{ΔL_1, ΔL_2}$, and -clpY$^{ΔL_1, +7 Gly}$, were separately transformed into yeast carrying pB42AD-clpQ$^{E61C}$; all the resultant transformants were selected on the SD-U-W-H media. These yeast co-transformants were then tested on leu-deficient media and streaked on X-gal media. As such, the transformants were blue on X-gal plates and grew well on the leu-deficient media. As shown, BD-ClpY$^{ΔL_1}$, -ClpY$^{ΔL_2}$, -ClpY$^{ΔL_1, ΔL_2}$, or -ClpY$^{ΔL_1, +7 Gly}$ was each associated with AD-ClpQ$^{E61C}$ in the two-hybrid assays, as was seen with the wild-type ClpY (Fig. 3A and 3B, lane 3, 4, 5 and 6 as compared to lane 2). To determine whether the C domain of ClpY is responsible for the association with ClpQ, the plasmid pGilda-clpYX encoding a chimeric protein ClpYX was constructed using the two-step PCR. Notably, ClpX did not associate with ClpQ; it formed a hexamer and its C domain (from S318 to E424 aa) had about 41% similarity to the ClpY counterpart (from T336 to L443 aa) (14). Therefore, a clpYX plasmid, with N and I domains of about 333 aa(s) encoded by the clpY* gene and the remaining 106 aa from the C domain (319–424 aa) of ClpX, was transformed with pB42AD-clpQ$^{E61C}$ into the yeast. Similarly, an earlier constructed pGilda-clpY$^{ΔC}$ (19) with a deletion of domain C was also transformed into EGY48 yeast carrying AD-ClpQ$^{E61C}$. The resulting transformants did not grow on leu-deficient media and were white on the X-gal media (in Fig. 3A and 3B, lane 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 an association with ClpQ.

ClpY$^{ΔL_1, +7 Gly}$, ClpY$^{ΔL_2}$, and ClpY$^{ΔL_1, ΔL_2}$ 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∆175−209, pBAD24-clpY∆L1, pBAD24-clpY∆L2, pBAD24-clpY∆L1, ∆L2 and pBAD24-clpYX. The E. coli strain AC3112, a lon clpQ clpY triple mutant, was thereafter used as a host cell for a co-transformation by pBAD33-clpQ+ in combination with pBAD24-clpY+ or its derivatives. Since both plasmids are compatible, the resultant co-transformant 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 methylmethansulfonate (MMS) at a low efficiency of plating (EOP, ≤10⁻³) and with higher β-galactosidase activity of cpsB::lacZ, due to an induction of SulA and the stability of RcsA in the absence of both Lon and ClpYQ proteases. After arabinose induction, bacterial cells that co-expressed 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 yeast that expressed ClpY∆L1, in the presence of ClpQ (Fig. 4A and 4B, lane 3). Bacterial cells that expressed either ClpY∆L2 or ClpY∆L1, ∆L2 or ClpY∆175−209 in the presence of ClpQ, showed higher β-galactosidase activity and increased MMS sensitivity as the negative control (Fig. 4A and 4B, lane 4, 5 and 6 as compared to lane 1). These results indicate that ClpY∆L1, ∆L2 and ClpY∆175−209 molecules were not able to bind to its substrates for the degradation by ClpQ. Since ClpY∆L1 is able to bind to the SulA, the loop 175−209 aa in ClpY, however, is apparently necessary for delivery of substrates.

The 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 media supplemented with ampicillin chloramphenicol and kanamycin. Each bacterial cell was then grown on LB with the appropriate antibiotics plus 0.5 % arabinose for the induction of ClpQ as well as ClpY and its derivative mutants. After bacterial cultures were grown to an early log-phase, IPTG (1mM at the final concentration) was subsequently added to the media; this led to the induction of HA-SulA. After 2 hours of induction, cell mixtures were diluted in series and plated for a calculation of efficiency of plating (EOP). Each bacterial strain had an EOP
colonies on media with IPTG induction divided by those without IPTG induction) similar to that determined by the corresponding MMS test (data not shown). Protein samples were also extracted and subjected to the 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, lane 1, line 1). Thus, the residual SulA was observed in different bacterial strains expressing ClpYΔL2, ClpYΔL1, ΔL2 or ClpYΔΙ +7Gly (Fig. 5, lane 4, 5 and 6; the first line). However, no SulA was observed in the bacterial strain that expressed wild-type ClpY or ClpYΔL1 in the presence of ClpQ (Fig. 5, lane 2 and 3; line 1). These results were consistent with the findings 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 the ClpYΔΙ +7Gly which has been expressed in a lower level (Fig. 5, line 2 and 3 through lane 2 to 6).

Random selection of ClpY mutants map in the 175–209 aa loop region and certain ClpY mutants defective on the degradation of substrates in the presence of ClpQ

To identify the critical amino acids that would 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 encompasses 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 pB42ADclpY+ (with an NruI-linearized clpY+ gene) mixed with a pool of the above PCR-mutated DNA fragments. The PCR products were then homologously recombined with the linearized pB42ADclpY+ plasmids. The resulting transformants were subsequently scored on X-gal indicator plates. Colonies were then selected with dark blue or white color. The pB42AD-clpY+ derivatives bearing mutations in the clpY+ structural gene were then extracted from the yeast cells, and the resulting extracted plasmids were retransformed into EGY48[p8op-lacZ] cells carrying pGilda-sulA+. The phenotypes of the newly transformed yeast were repeatedly confirmed on both the X-gal media and the leu-deficient plates. The sites of mutations in 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 (as shown in Fig. 1). As compared to the positive control (yeast carrying AD-ClpY/BD-SulA), yeast that expressed BD-ClpY<sub>M187I</sub> and AD-SulA were grown on leu-deficient media and were dark blue on X-gal media with higher β-galactosidase activity (Fig. 6A and 6B, lane 3, as compared to lane 2). The ClpY<sub>L199Q</sub>, however, showed the least binding activity toward SulA; the yeast cells were white on the X-gal plate and expressed a lower β-galactosidase activity, and cell growth was barely observed on leu-deficient media (Fig. 6A and 6B, lane 5). Yeast, expressing ClpY<sub>A188S</sub> or ClpY<sub>N205K</sub> 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 6B, lane 4 and 6). Similar phenotypes were observed when BD-SulA<sub>M89I</sub> was expressed in yeast (Fig. 6A, lane 2 to 6). In addition, using the anti-HA antibody, AD-ClpY and its derivatives were observed in the yeast (Fig. 6C, lane 2 to 6).

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

The residual SulA and ClpQ, as well as ClpY and its derivatives, were then simultaneously detected in the above bacterial strains. SulA accumulated in the bacterial cells that expressed ClpY<sub>L199Q</sub> (Fig. 6E, lane 5; line 1). Little or no SulA accumulated in cells that expressed ClpY<sub>M187I</sub>, ClpY<sub>A188S</sub> or ClpY<sub>N205K</sub> (Fig. 6E, lane 3, 4 and 6; line 1). ClpQ was expressed equally in each of the bacterial strains (Fig. 6E, lane 2 to 6; line 2), and ClpY and its mutant derivatives were each equally expressed in all the bacterial strains (Fig. 6E, lane 2 to 6; line 3).

The site-directed ClpY<sup>E193L, E194L</sup> and ClpY<sup>Q198L, Q200L</sup> loop 2 mutants, with an enhanced or a moderate SulA-binding activity, but with no degradation by ClpQ

To further confirm that the hydrophobic side chains of aa 175–209 in ClpY take part in the binding of substrates, the site-directed mutagenesis was used to construct single or double point mutations in the same region. These site-specific ClpY mutants have the substituted residues at a position(s) adjacent to those of the randomly selected one(s). The clpY<sup>H186N</sup>, clpY<sup>E193L, E194L</sup> and
clpYQ198L, Q200L were made in pB42AD plasmids subsequently designated as pB42AD-clpY1186N, pB42AD-clpYE193L, E194L and pB42AD-clpYQ198L, 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-sulA89M, and the resultant transformants were scored on X-gal plates, measured of the β-galactosidase activity, and tested on the leu-deficient media.

ClpY198L, Q200L and ClpY1186N, E193L, E194L were significantly associated with SulA/SulA89M (Fig. 7A, and 7B, lane 4 and 5), whereas ClpY1186N did not interact with SulA when compared to the wild-type ClpY (Fig. 7A and 7B, lane 3 as compared to lane 2). Thus, these results suggest that the hydrophobic groups of amino acids in the loop of ClpY are necessary for its binding activity towards the substrates. The AD-ClpY and its derivative mutants were expressed well in the yeast (Fig. 7C, lane 2 to 5).

When AC3112 cells co-expressed ClpQ with ClpY1186N, ClpYE193L, E194L or ClpYQ198L, Q200L, each a bacterial strain showed the MMS sensitivity and had the higher β-galactosidase activity of cpsB::lacZ (Fig. 7D, lane 3 to 5) as compared to cells with the wild-type ClpY (SmaI) (Fig. 7D, lane 2 and lane 1 as a negative control). Therefore, these ClpY mutants showed a 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 are shown in Fig 7E. Coincident with the MMS findings, SulA was accumulated in cells that expressed ClpY1186N, ClpYE193L, E194L or ClpYQ198L, Q200L (Fig 7E, line 1; lane 3 to 5) but not in cells that express the wild type ClpY (SmaI) (Fig 7E, line 1; lane 2). It is likely that ClpY1186N is defective in substrate binding whereas ClpYE193L, E194L and ClpYQ198L, Q200L are defective in an intracellular delivery of substrates for the further degradation by ClpQ.

The clpYN141L, N142L, a loop 1 mutant, defective in complete degradation of SulA

In addition, to assess whether the residues in L1 loop are involved in delivery activity, clpYN141L, N142L and clpYQ148L, Q149L, Q150L, in which both clpY genes carry multiple mutations in the L1 loop, were made and the mutated genes were cloned into pB42AD and designated pB42AD-clpYN141L, N142L and pB42AD-clpY148L, Q149L, Q150L, respectively. After transformation of pB42AD-clpYN141L, N142L and pB42AD-clpY148L, Q149L, Q150L with pGilda-sulA+/sulA89M, all the co-transformants were scored on X-gal plates and tested on the leu-deficient media. The yeast cells
were blue on the X-gal media with higher β-galactosidase activity than the control and grew on
the leu-deficient media (Fig. 8A and 8B, lane 2, 3 and 4). As indicated, ClpY_{N141L, N142L} and
CpY_{Q148L, Q149L, Q150L} significantly interacted with SulA/SulA_{M89I} as compared to the wild-type
ClpY. Both ClpY_{N141L, N142L} and CpY_{Q148L, Q149L, Q150L} expressed well in yeast as did the wild-
type ClpY (Fig. 8C, lane 3 and 4 as compared to the lane 2). While the AC3112 bacterial cells
that co-expressed ClpQ with ClpY_{N141L, N142L} was MMS sensitive and had subtly increased β-
galactosidase activity from the cpsB::lacZ as compared to the wild-type ClpY (Fig. 8D, lane 3,
as compared to lane 2). The ClpY_{Q148L, Q149L, Q150L}, however, showed the normal activity similar
to the wild-type ClpY (Fig. 8E, lane 4, as compared to lane 2). In the presence of ClpQ, SulA
accumulated in AC3112 cells that expressed the ClpY_{N141L, N142L}, but not in the cells that
expressed ClpY{Q148L, Q149L, Q150L} (Fig. 8E, lane 3 and 4, as compared to the lane 2). The ClpQ and
ClpY with its derivatives were expressed in all bacterial strains (Fig. 8E, lane 2 to 4). Therefore,
the ClpY_{N141L, N142L} mutant appeared to be defective in substrate delivery for the degradation by
ClpQ.
DISCUSSION

One of the specific aims of this study was to determine whether domain I of ClpY is responsible for recognition of its natural substrates. Using a ClpYΔI+7Gly mutant that lacked the I domain but contained 7 Gly residues as a hinge between the N and C domains, we showed that the domain I is required for association with SulA in the yeast two-hybrid assays. In addition, ClpY and ClpYΔI+7Gly mutants both interacted with ClpQE61C. Thus, the protruding domain I of ClpY functions in cellular target recognition. However, neither ClpYX, with a C domain substituted from ClpX, nor a ClpYAC mutant, completely lacking the C domain, associated with ClpQE61C. 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 (175-209 aa) in the I domain of ClpY is required for the degradation of MBP-SulA by ClpQ (33). The 175th to the 209th residues constitute an α loop in the X-ray structure of the analogous ClpY of H. influenza (34). Here, a ClpYΔL2 mutant, lacking aa 175-209, was shown to associate with SulA. Accordingly, ClpYΔL1, with a deletion of 137-150 aa, has slightly less proteolytic activity for MBP-SulA (33). ClpYΔL1, ΔL2 did not associate with SulA, and in the presence of ClpQ, the mutant complex led to no degradation of SulA or RcsA. However, ClpYΔL1 and ClpYΔL2 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 the loop 2 (L2) in ClpY was shown to have much stronger binding to SulA, and ClpYΔL2 could not degrade SulA in the presence of ClpQ, it is likely that this loop (175-209 aa) 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 175-209 aa loop region. Interestingly, the substituted amino acids such as ClpYA188S, ClpYI186N, ClpYL199Q, and ClpYN205K with polar or more positively charged side groups led to less SulA-binding activity. However, ClpYL199Q and ClpYI186N, both molecules were expressed at lower level in the yeasts. It is our previous result that AD-ClpY was not so easily detected if no interactive protein was present (19). This observation also supports that ClpYL199Q showed the least binding activity and ClpYI186N displayed no binding to SulA. Conversely, hydrophobic groups in the side chains of the substituted residues, e.g. ClpYM187I led to enhanced SulA-binding. Thus, the hydrophobic side chains of the residues in
the 175-209 aa loop are likely involved in an inter-modular association between the chaperone and its substrates.

Both ClpY\textsuperscript{E193L, E194L} and ClpY\textsuperscript{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 toward the central pore of the cylindrical complex. In contrast, ClpY\textsuperscript{M187I} showed higher SulA-binding activity, and the substrates were degraded in the presence of ClpQ. Moreover, ClpY\textsuperscript{A188S} and ClpY\textsuperscript{N205K} showed slightly less binding to SulA, and only the ClpY\textsuperscript{N205K} mutant showed a minimal decrease in substrate degradation in the presence of ClpQ. Therefore, ClpY\textsuperscript{M187I}, ClpY\textsuperscript{A188S} and ClpY\textsuperscript{N205K} mutants maintained their ability to deliver substrates for degradation by ClpQ. Interestingly, a small amount of residual SulA was routinely detected when ClpY\textsuperscript{N205K} and ClpQ were expressed in the protease-complex suggesting that the substrates were being progressively degraded. Additionally, the loop 1 mutant, ClpY\textsuperscript{Q148L, Q149L, Q150L}, binds to SulA, but it does not efficiently degrade the protein. As well, with this mutant protein in AC3112 that has ClpQ, it is always shown a slightly increased β-galactosidase activity of \textit{cpsB::lacZ} as 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 the delivery activity of ClpY demonstrated here occurred likely 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\textsuperscript{2+} (3). The nucleotide-bound ATP(s)-ClpY molecules are responsible for the recognition of substrates in the ClpYQ complex. It was also later shown that three or four ATP (s) 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, 137-150 aa and 175-209 aa, in domain I of ClpY are essential for association of the natural substrates and that the 175-209 aa loop is likely involved in gripping the substrates. But, 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\textsuperscript{ΔL1} or ClpY\textsuperscript{ΔL2} showed enhanced binding to SulA\textsuperscript{M89I}, suggesting that other binding sites in domain I in the above mutants could be involved in substrate tethering. Accordingly, we
identified a couple 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 (137-150aa) and the inner (175-209 aa) loop, function in cellular substrate binding and that the inner loop is responsible for the gripping of natural substrates such as SulA and possibly for intra-cylindrical delivery. However, the outer loop (137-150 aa) also may 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, which 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 in accord 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, delivering, 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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FIGURE LEGENDS:

Fig. 1. Domains of ClpY and its functional motifs as well as its derivative mutants. 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 57~66) and BoxB (aa 253~256 aa), the nucleotide binding pocket (aa 17~19, aa 57~66 and aa 80~89), the domain I tip (aa 154~165) and two loops (aa 137~150 and aa 175~209), pore I (aa 89~94) and pore II (aa 264~269). The selective ClpY mutants and the site-specific-substituted ClpY mutants are in the square boxes. The original nucleotides of the amino acids and those of the substituted mutants are shown.

Fig. 2. Expression of lacZ and LEU2 in EGY48[p8op-lacZ] with an each pair of AD-/BD- fusion proteins. The BD domain is from pGilda and the AD domain is from pB42AD. Each fusion protein was as indicated. Panel (A), expression of lacZ, with colony color evaluated on Gal+Raf-Ura-His-Trp plates containing X-Gal over 4 days. The 1st line is the control, in which yeasts express BD-ClpY or its derivative mutants with an AD domain. The 2nd line is the BD-ClpY and its derivative mutants with an AD-SulA. The 3rd line is the ClpY and its derivative mutants with SulA^{M89I}. Panel (B), lane 1 is a reciprocal negative control and lane 2 is AD-ClpY with BD-SulA/SulA^{M89I}. Panel (C), the β-galactosidase activity of the yeasts carrying BD-ClpY or its derivatives/AD-SulA or SulA^{M89I}. Panel (D), LEU2 expression of the yeasts on Gal+Raf-Ura-His- Trp-Leu plates over 4 days. The 1st line is the control as described as above. The 2nd line is the yeasts, grown on the leu-deficient plates, expressing a pair of BD-ClpY or its derivative mutants with an AD-SulA. Panel (E), Western blotting of ClpY and its derivatives in the BD-fusion protein using an anti-LexA antibody.

Fig. 3. Expression of lacZ and LEU2 in EGY48[p8op-lacZ] cells carrying ClpY and its derivative mutants with ClpQ^{E61C}. Panel (A), the 1st line is the control yeasts on X-gal plates over 4 days. The 2nd line illustrates the interaction between ClpY and its derivative mutants with ClpQ^{E61C}. Panel (B), LEU2 expression of the yeasts on Gal+Raf-Ura-His-Trp-Leu plates over 4 days. The 1st line is the control. The 2nd line is an each strain carrying a pair of BD-ClpY or its derivative mutants with AD-ClpQ^{E61C}. Panel (C), Western blotting of ClpY and its derivatives and ClpQ^{E61C} using anti-LexA and anti-HA antibody, respectively.

Fig. 4. A chart of cpsB::lacZ expression and MMS tests for an 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 0.5~0.9, 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 ratio of the average number of colonies formed on media with 0.025 % MMS divided by the average number of colonies detected on media without an addition of 0.025 % MMS. Bacterial cells carrying pBAD33 and pBAD24 were used as a negative control.
Fig. 5. Residual HA-SulA and ClpQ as well as ClpY and its derivatives for each bacterial strain. Cells were grown to OD$_{600} = 0.3$ in LB with addition of appropriate antibiotics and 0.5% arabinose; then 1 or 2.5 mM IPTG was added into the media for induction of HA-SulA for 2 hrs. Afterwards, samples were extracted, normalized with the OD$_{600}$, and loaded as described in the Materials and Methods. The 1st line shows the residual SulA, the 2nd line shows the ClpQ, and the 3rd line 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. Panel (A), the assays of the β-galactosidase activity in the yeasts; each pair of AD-/BD- fusion protein as indicated. The 1st line, is the control and the 2nd line is the AD-ClpY/its derivatives and BD-SulA and the 3rd line is the AD-ClpY/its derivatives and BD-SulA$_{M89I}$. The yeasts were grown on the leu-deficient media (C). The 1st line, is the controls and the 2nd line, is the AD- ClpY or various ClpY mutants with BD-SulA. Panel (D), Western blotting of ClpY and its derivatives in the AD-fusion protein using an anti-HA antibody. The assays of $cpsB::lacZ$ expression and MMS tests for each bacterial condition (E). Each bacterial strain carries the wild-type ClpY or the various ClpY mutants, in the presence of ClpQ. The top panel is the β-galactosidase level of the $cpsB::lacZ$ expression of each bacterium. The bottom line is the number of the EOP for each bacterial condition in the MMS tests. Western blots of the ClpY, ClpQ and HA-SulA of each bacterium (F). The 1st line is, as indicated, the HA-SulA, the 2nd line is, as indicated, ClpY and its derivative mutants and the 3rd line is ClpQ, as indicated.

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

Fig. 8. Expression of lacZ and LEU2 in EGY48[p8op-lacZ] cells carrying different ClpY loop 1 mutants in the AD domain as well as AD-ClpY (SmaI) for an interaction with BD-SulA/SulA$_{M89I}$. The physiological activities in each bacterial condition are shown. Yeasts were measured of the β-galactosidase activity with each pair of AD-/BD-fusion proteins as indicated (A) or grown on the leu-deficient media (B). Western blotting of AD-ClpY (SmaI) and various AD-ClpY mutants in the yeasts using an anti-HA antibody is shown (C). The $cpsB::lacZ$ assays and MMS tests for each bacterial condition carrying the wild-type ClpY or its derivative mutants with ClpQ are shown in (D). Western blots of HA-SulA, and ClpY and its derivatives as well as ClpQ in the bacterial strains are shown (E).
REFERENCES

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

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<td></td>
<td>P137Q, Q150L</td>
<td>C102L, C104L</td>
</tr>
<tr>
<td></td>
<td>R138Q</td>
<td>G102L, G104L</td>
</tr>
</tbody>
</table>

### Notes:
- a. the ClpY mutants constructed by the site-directed mutagenesis
- b. the original sequence of the amino acid
- c. the substituted sequence of the mutants