

Escherichia coli Proteome Microarrays Identified the Substrates of ClpYQ Protease*[§]

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Proteolysis is a vital mechanism to regulate the cellular proteome in all kingdoms of life, and ATP-dependent proteases play a crucial role within this process. In *Escherichia coli*, ClpYQ is one of the primary ATP-dependent proteases. In addition to function with removals of abnormal peptides in the cells, ClpYQ degrades regulatory proteins if necessary and thus let cells adjust to various environmental conditions. In *E. coli*, SulA, RcsA, RpoH and TraJ as well as RNase R, have been identified as natural protein substrates of ClpYQ. ClpYQ contains ClpY and ClpQ. The ATPase ClpY is responsible for protein recognition, unfolding, and translocation into the catalytic core of ClpQ. In this study, we use an indirect identification strategy to screen possible ClpY targets with *E. coli* K12 proteome chips. The chip assay results showed that YbaB strongly bound to ClpY. We used yeast two-hybrid assay to confirm the interactions between ClpY and YbaB protein and determined the K_d between ClpY and YbaB by quartz crystal microbalance. Furthermore, we validated that YbaB was successfully degraded by ClpYQ protease activity using ClpYQ *in vitro* and *in vivo* degradation assay. These findings demonstrated the YbaB is a novel substrate of ClpYQ protease. This work also successfully demonstrated that with the use of recognition element of a protease can successfully screen its substrates by indirect proteome chip screening assay. *Molecular & Cellular Proteomics* 16: 10.1074/mcp.M116.065482, 113–120, 2017.

Protease is responsible for protein degradation that can digest long protein chains into shorter fragments by splitting the peptide bonds (1). Protease exists in a wide range of organisms. One of the most important functions for protease is to degrade the misfolding or functionless proteins. Misfolding or functionless proteins usually lead to severe diseases,

such as Alzheimer's disease, bovine spongiform encephalopathy, cardiovascular disease, cancer, osteoporosis, neurological disorders, and type II diabetes (2–4). In addition, the protease-induced protein regulation is very important in the wide-range organism. Protease-induced protein regulation in bacterial cells is related to the antibiotic resistance, environmental resilience, and infectivity of bacteria (5). Altering the growth environments of bacteria (e.g. adjusting temperature, osmotic pressure, nutrient deficiency, ultraviolet irradiation, addition of antibiotics or other chemicals) can induce abnormal protein synthesis and accumulation in bacterial cells. Under such conditions, external environmental stimuli can activate the response systems of bacteria, including heat-shock and SOS responses, which can trigger the biosynthesis of the corresponding proteases, potentially aiding bacteria in overcoming unfavorable growth conditions (6, 7). The ATP-dependent protease of *E. coli* provides us an excellent example to understand the importance of protein degradation role in cell physiology. The homologs of these energy (ATP or GTP)-dependent proteases were also found in eukaryotes indicating the similar regulation cascades between prokaryotes and eukaryotes (5). There are four important ATP-dependent proteases in prokaryotes, Lon, FtsH, ClpAP/XP, and ClpYQ (8). Lon protease is a major cytosol protease in *E. coli*, which is a DNA heat shock protein responsible for degradation of misfolding proteins (9, 10). FtsH is a membrane-bound essential protease, which degrades the heat-shock transcription factor sigma 32 (11). The ClpAP/XP and ClpYQ belong to the Clp protease family. The *clp* stands for caseinolytic protease, which can degrade casein *in vitro* (12, 13). ATP-dependent protease ClpYQ is a type of protease complex. The translational products of *clpQ*⁺*Y*⁺ operon are ClpQ (HslV), the peptidase responsible for proteolysis, and ClpY (HslU), an ATPase with unfolding activity (14). ClpYQ was suggested as an alternative protease to partially restore the function of Lon protease (15, 16). Bochtler *et al.* (17) reported the protein crystal structure of ClpYQ, which is composed of ClpY and ClpQ hexamers (ClpY₆ and ClpQ₆). The centers and both ends of two ClpQ hexamers are each connected to a ClpY hexamer, thereby forming a ClpYQ complex (18–20). Previous studies (17, 20) have verified that ClpY can only form a ring complex under the presence of nucleotides such as of ATP, ADP, and nonhydrolysable ATP analogs (e.g.

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AMP-PNP or ATP- γ -S). By contrast, ClpQ can spontaneously form a six-membered ring. Therefore, synthesis of a complete ClpYQ complex must involve nucleotides including ATP (21). A previous study (17) divided the structure of ClpY into three domains: the N-terminal domain (N domain, Ser2-Lys109, Ile244-Leu332), intermediate domain (I domain, Met110-Ala243), and C-terminal domain (C domain, Gln333-Leu443). The N domain comprises ATP binding sites. The I domain is comparatively further away from ClpQ than the N domain is and contains a double-loop structure for recognizing and tethering substrates. The C domain involves aggregating the atoms in the ClpY and ClpQ complexes (22–24). Previous studies on how ClpY recognizes substrates have revealed that the G⁹⁰Y⁹¹V⁹²G⁹³ pore motif (pore 1 site) located at the center pore of ClpY is conservative and essential for unfolding and transporting substrates (25–27).

Currently, few substrates have been identified for the ClpYQ protease in comparison with other ATP-dependent proteases (25–27). Because the substrate of a protease can reflect its role in a bacterial cell, identifying potential enzyme substrates is essential in protease research. Using substrate-enzyme interactions as indicators for screening enzyme substrates in large batches has become a prevalent approach in recent studies on ATP-dependent protease substrates (28, 29). Because ClpY is responsible for substrate recognition, in this study, we used *E. coli* proteome microarrays to identify ClpY recognition proteins and the results showed that the ClpY exhibited strong interactions with the YbaB. We also used a yeast two-hybrid assay system to validate the interactions between ClpY and YbaB. Additionally, a quartz crystal microbalance (QCM)¹ was used to measure the K_d values of YbaB-ClpY interactions. The *in vitro* and *in vivo* degradation tests were conducted to confirm the degradation of YabB by ClpYQ. These results proposed the YabB is a novel target of ATP-dependent protease ClpYQ.

EXPERIMENTAL PROCEDURES

Fabrication of the *E. coli* K12 Proteome Chips—For the studies of the bacterial proteome, we have constructed the *E. coli* K12 proteome microarray (30). In short, the *E. coli* K12 ASKA library (31) was first incubated with 2 × Luria Broth (LB) medium containing 30 μ g/ml chloramphenicol at 37 °C overnight. Then, the overnight cultures were diluted with 2 × LB to an OD₅₉₅ value of 0.1. When the OD₅₉₅ value reached ~0.8, isopropyl β -D-thiogalactoside was added to the culture and incubated at 37 °C for ~2 h. The culture was harvested by centrifugation, and the pellets were stored at –80 °C before purification.

To purify the proteins, the frozen cell pellets were thawed on ice and re-suspended in lysis buffer consisting of 50 mM NaH₂PO₄, 300 mM NaCl, 30 mM imidazole, CelLyticB, 1 mg/ml lysozyme, 50 units/ml benzonase, proteinase inhibitor mixture, 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MA), and Ni-NTA Superflow resins (Qiagen, Hilden, Germany) at 4 °C. After 2.5 h incubation on the plate shaker at 4 °C, the mixtures were transferred into 96-well filter plates (Nunc). The plates were then washed with wash buffer I (50 mM

NaH₂PO₄, 300 mM NaCl, 20% glycerol, 20 mM imidazole, and 0.1% Tween 20) and with wash buffer II (50 mM NaH₂PO₄, 150 mM NaCl, 30% glycerol, 30 mM imidazole, and 0.1% Tween 20, pH 8, Sigma-Aldrich). Finally, the proteins were eluted with elution buffer (50 mM NaH₂PO₄, 150 mM NaCl, 30% glycerol, 300 mM imidazole, and 0.1% Tween 20, pH 7.5). After the purified proteins were prepared, they were printed in duplicate on aldehyde slides by ChipWriter Pro (Bio-Rad, Hercules, CA) with 48 pins in a cold room. The chips were then stored at –80 °C before utilization.

The *E. coli* K12 Proteome Chip Assays—To reduce nonspecific binding, the chip was blocked with 1% bovine serum albumin. 0.5 μ M ClpY (27) or ClpY (Δ I+Gly) (22, 27) in TBS-T (Tris-buffered saline contains 0.05% Tween 20) contains 5 mM ATP and 1% bovine serum albumin were used to probe the chips in a hybridization chamber with shaking at room temperature for 1 h. After incubation, the chips were washed with TBS-T, and incubated with anti-ClpY antibody (22) for another 1 h. After washed with TBS-T again, DyLight 649 (Thermo Scientific, Waltham, MA) labeled goat anti-rabbit antibodies (Abcam, Cambridge, England) were used to probe the chips at room temperature for 30 min. Finally, the chips were washed several times with TBS-T and distilled water. After the final wash, the chips were dried by centrifugation at 201 × *g* and then scanned with a microarray scanner (Axon GenePix 4000B).

Bioinformatics Analysis of the Chip Assays—To process the images of the chip assay results, GenePix Pro 6.0 was used to align each protein spot and export all of the images to text files. For data preprocessing, ProCAT (32) was applied to normalize the signals and hit selection. Positive hits were selected based on a local cutoff, which was defined as triple standard deviations above the signal mean for each spot.

Construction of YbaB and ClpY Protein Expression Plasmids for Yeast Two-Hybrid, In Vitro and In Vivo Assay—The primers used for YbaB gene amplification, restriction site generation and his-tag fusion for expression plasmid construction are listed in supplemental Table S1. pGilda and pB42AD (Clontech®, Mountain View, CA) vector plasmids were chosen to construct pB42AD-*ybaB* and pGilda-*ybaB* for later yeast two hybridization assay. The YbaB expression plasmid (pB42AD-*ybaB* or pGilda-*ybaB*) was constructed using the restrictive enzymes *EcoRI* and *XhoI* sites in the two primers and the template from the chromosomal DNA of MG1665; subsequently, the *ybaB* gene fragment were amplified using polymerase chain reaction (PCR) and then ligated to pB42AD or pGilda vectors, which were prepared using the same restrictive enzymes. Next, target gene constructed in the pB42AD and pGilda plasmid were transformed into the XL-1 Blue *E. coli* strain, and a selection medium with ampicillin was then used to screen for the transformed bacterial colonies contained the *ybaB* gene. The *clpY* gene expression plasmids pB42AD-*clpY* and pGilda-*clpY* have already been constructed by Lee *et al.* (23).

The restrictive enzymes *EcoRI* and *HindIII* were used to cleavage the gene from pB42AD-*ybaB* plasmid, and the gene was then ligated to pMal-c2x (NEB®) and pTH18kr-*mbp* vectors (35), which were prepared using the same restrictive enzymes, to construct pMal-c2x-*ybaB*, and pTH18kr-*mbp-ybaB*. pET21a-(6x his tag)-*clpY* and pET21a-*clpQ*-(6x his-tag), the expression vectors used for protein purification of His(6x)-ClpY and ClpQ-His(6x) were constructed by Hsieh *et al.* (27). The vectors were transformed into *E. coli* and then screened using a growth medium containing ampicillin (pTH18kr-*mbp* was screened by kanamycin). Those strains with different plasmids will be stored until required for later validation assay.

Yeast Two-hybrid Assay—Two plasmid sets, pGilda-*ybaB* and pB42AD-*clpY* or pGilda-*clpY* and pB42AD-*ybaB*, were co-transformed into EGY48 yeast strain to conduct the yeast two-hybrid assays. Interactions between the proteins expressed by the two

¹ The abbreviations used are: QCM, quartz crystal microbalance.

plasmids were verified by observing whether the *lacZ* reporter gene in the transformed strain was activated. The EGY48 yeast strain was transformed by first culturing the yeasts in a 5-ml SD/Ura medium at 30 °C for 12–14 h until the OD₆₀₀ reached to 0.4–0.6. Then yeast cells were centrifuged and washed with sterile water and 100 mM lithium acetate (LiOAc) and resuspended in 240 μl of 50% (v/v) polyethylene glycol (PEG). Mixing 36 μl of 1 M LiOAc, 25 μl of salmon sperm DNA (before use, this reagent was boiled at 100 °C and placed in an ice bath for 10 min), with 50 μl of each plasmid and added to the yeast cell suspension. The mixture was vigorously shaken for 1 min, incubated at 30 °C for 30 min, followed by heat-shock treatment in a 42 °C water bath for 20–25 mins. After heat-shock, the solution was centrifuged at 14,000 rpm for 5 s, and the supernatant was removed. The yeast cells were suspended in 100 μl of sterile water and then smeared on growth plates containing selective growth media (S.D./-Ura/-His/-Trp). After incubated for 2–3 days at 30 °C, a transformed yeast colony was transferred to a new S.D.-Ura/-His/-Trp growth plates at 30 °C for 1–2 days incubation to activate the cells. The activated cells were spread on 80-mg/L X-gal (Gal/Raf/-Ura/-His/-Trp) growth plates and incubated at 30 °C for 2 days to observe the cell growth.

Measurement of K_d Between MBP-YbaB and ClpY—A QCM (Affinix QN μ) was employed to measure the K_d values of the YbaB-ClpY protein interactions, which were expressed in the transformed *E. coli* cells through pMal-ybaB and pET21a (6x *his* tag)-*clpY* (27). The MBP-YbaB and His-ClpY were purified using amylose resin (New England Biolabs, NEB, Ipswich, MA) and Nickel resin (Talon resin, Takara, Kusatsu, Shiga, Japan), respectively. In the beginning, the sensor cell of the QCM was cleaned in a 500-μl sodium dodecyl sulfate (SDS, 1%) solution and then dried with compressed nitrogen gas. Subsequently, the sensor cell was merged with 300 mM HEPES buffer containing 30 mM MgCl₂, 3 mM DTT, and 3 mM EDTA, and the 8 μl of MBP-YbaB (1 mg/ml) was immobilized on electrodes. After the frequency of the sensor was decreased and remained stable, 5 μl of MBP-YbaB was added again to confirm the frequency remain stable, indicating that MBP-YbaB already saturated on sensor cell. Then 2 μl of bovine serum albumin (BSA) solution was added to reduce non-specific binding. The sensor cell was washed twice with 450 μl of HEPES buffer to remove the unbound MBP-YbaB and BSA. Before adding the next sample, the sensor cell was filled with 450 μl of HEPES buffer and stand for 3 min to stabilize the frequency. Finally, 1, 3, 5, 8, and 10 μl samples of ClpY protein (10 ng/μl) were separately loaded into the sensor cell to measure the decrease in frequency. AQUA analysis software (Initium Co., Chigasaki, Kanagawa, Japan) was employed to calculate the K_d values.

MBP-YbaB In Vitro Degradation Test—Two μM ClpY, 2 μM ClpQ, and 1 μM MBP-YbaB were mixed with 15 μl of 4X degradation solution to create a 60-μl reaction solution. The reaction solution was incubated at 37 °C. After incubation started, each 20 μl of the reaction solution was collected at 0, and 4 h, respectively. An equal volume of 2× SDS protein sample buffer solution was added to each reaction solution and mix by vortex. The mixture was boiled at 100 °C for 5 min to denature the protein and inactive protease activity. The mixture containing ClpY, ClpQ, and MBP-YbaB was analyzed by SDS-PAGE and then stained with Coomassie blue R-250. The image was scanned by DigiGel® scanner. The degradation of MBP-YbaB proteins was also detected by Western blotting with anti-MBP antibodies. The Western blot membrane was exposed to autoradiography film (Classic Blue, MIDSCI). The signal intensity was used to calculate YbaB protein degradation by using Image J software. Means and standard deviations were taken from three independent analyses and the significant differences were calculated by Student's *t* test ($p < 0.05$).

MPB-YbaB In Vivo Degradation Assay—The pTH18kr-*mbp-ybaB* plasmids were transformed into *E. coli* SG22623 (+ClpYQ) and AC3112 (-ClpYQ) strains. The transformed bacterial cells were cultured in LB broth until the OD₆₀₀ reached to 0.3–0.4, and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to induce protein expression for 30 min. Subsequently, spectinomycin (at final concentration, 100 μg/ml) was added to terminate the protein expression. Next, the OD₆₀₀ values of the culture medium were recorded at 0, and 240 mins. Bacterial cells were also harvested at these time points. The harvested cells were resuspended and centrifuged in sterilized water to remove the growth media solution. An equal volume of 2× SDS protein sample buffer was added to the samples and boiled at 100 °C for 5 mins. The degradation of MBP-YbaB proteins was detected by Western blotting with anti-MBP antibodies. The Western blot membrane was exposed to autoradiography film (Classic Blue, MIDSCI) and the image signal intensities were used to calculate YbaB protein degradation by using Image J software. Means and standard deviations were taken from three independent analyses and the significant differences were calculated by Student's *t* test ($p < 0.05$).

RESULTS

The *E. coli* Proteome Chip Assays and Bioinformatics Analysis—We employed a high-throughput *E. coli* proteome microarray to systematically identify the recognition proteins of ClpY. The *E. coli* proteome microarrays were composed of ~4300 nonredundant proteins, which were printed in duplicated on aldehyde-coated glass slides as previously reported (30). In this study, the *E. coli* proteome chips were probed with ClpY and ClpY(ΔI+7Gly) proteins. ClpY(ΔI+7Gly) is an I-domain deletion mutant protein from ClpY but maintains the configuration, which lost its binding ability to substrate (22, 33). In this study, we use ClpY(ΔI+7Gly) as a control for identifying specific ClpY recognition proteins. Then the rabbit anti-ClpY detection antibodies were used to recognize ClpY on the chip (Fig. 1A). At last, DyLight™ 649 labeled anti-rabbit antibodies were used to detect anti-ClpY and show fluorescent signals on the chips. After the washing steps, the chip was scanned and analyzed. A representative chip image comparison between ClpY and ClpY(ΔI+7Gly) was shown in Fig. 1B. The ClpY and ClpY(ΔI+7Gly) chip images indicated different binding affinity between particular proteins. The representative proteins YfbV, RhlB, and YbaB showed stronger signal in ClpY than ClpY(ΔI+7Gly), respectively (Fig. 1B). This indicated ClpY recognize with specific targets on *E. coli* chips. After conducting chip analysis, the signal was output as a text file for signal normalization and positive hit selection. Each signal was normalized with ProCAT (32). The distribution of the signal was fit to a normal distribution, and the positive hits were selected based on a local cut-off, which was defined as triple standard deviations above the signal mean for each spot. After the initial selection, we chose the possible candidates only bind to ClpY, but not ClpY(ΔI+7Gly). After eyeballing, we remove 3 of 9 hits because of not obvious difference between control and experiment. Thus, 6 proteins were identified and summarized in Table I.

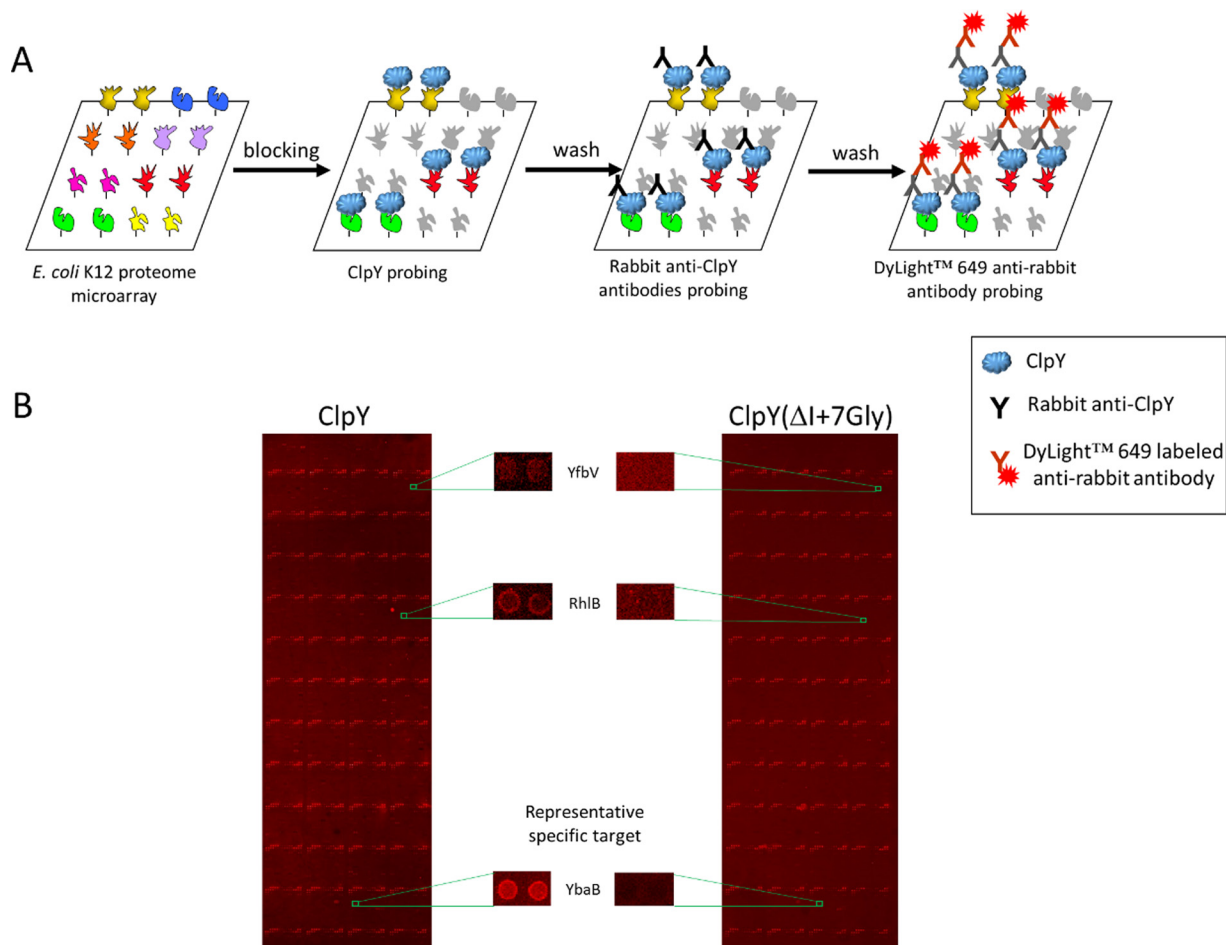


FIG. 1. **A** schematic representation of ClpY recognition protein identification using an *E. coli* proteome chip. **A**, The ClpY proteins were probed to the *E. coli* proteins on the chips and detected by anti-ClpY antibodies. The anti-rabbit antibodies were labeled with DyLight™ 649. **B**, Representative images of the *E. coli* proteome chips probed with ClpY and control ClpY($\Delta I+7Gly$). The representative proteins of YfbV, RhlB and YbaB on the chips were enlarged from sample images of ClpY and control ClpY($\Delta I+7Gly$), respectively.

TABLE I
Six ClpY-interacting proteins selected by *E. coli* K12 proteome chips

Accession number	Protein name	Annotation	Signal intensity
EG11100	YbaB	DNA-binding protein	667.1 \pm 34.3
EG14106	YfbV	Ter macrodomain insulation protein	218.4 \pm 31.5
EG10817	RbsD	D-ribose pyranase	140.6 \pm 20.5
EG13443	CobB	Protein lysine deacetylase, chemotaxis regulator	114.8 \pm 44.6
EG10844	RhlB	ATP-dependent RNA helicase	100.8 \pm 71.0
EG13114	YgbT	Multifunctional endonuclease Cas1, CRISPR adaptation protein	82.7 \pm 7.7

Yeast Two-hybrid Assay Analysis—Because YbaB signal is much higher than other 5 proteins, we focused on YbaB for further validations and studies. First, we used yeast two-hybrid system to validate the interaction between YbaB and ClpY. The yeast two-hybrid assay system involved in transforming the target protein gene segments into pB42AD and pGilda plasmids, which can express the target active domain (AD) and binding domain (BD) fusion proteins, respectively. We cloned *ybaB* into pGilda plasmids, which were co-transformed along with pB42AD-*clpY* into EGY48 yeast cells. To

account for the effect of AD or BD fusion on the target proteins, *ybaB* genes was cloned into pB42AD plasmids and cotransformed along with pGilda-*clpY* into EGY48 yeast cells. pGilda/pB42AD plasmids with and without the cloned genes were cotransformed into yeast cells. Concurrently, we verified whether the target proteins interacted with B42 peptide (the product of pB42AD) or LexA (the product of pGilda). Cotransformation of the pGilda and pB42AD plasmids without the cloned genes yielded the negative control group. To perform the X-gal test, the yeast two-hybrid assay analysis was con-

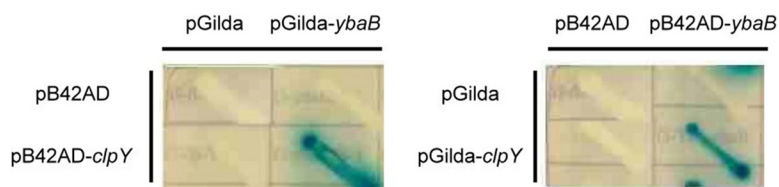


FIG. 2. **Interactions between ClpY and YbaB in the yeast two-hybrid assay.** The vector-only pB42AD and pGilda were cotransformed as a negative control. Interactions between ClpY and YbaB protein were tested by streaking the yeast colony on X-Gal containing minimal medium with an addition of raffinose and galactose. Turning blue of the colony indicates interactions.

ducted using the reporter plasmid p8op-*lacZ* carried by the EGY48 yeast cells. The X-gal test results revealed obvious YbaB-ClpY reactions (Fig. 2). The interaction between expressed ClpY and YbaB caused β -galactosidase expression and further degraded the X-gal compounds, forming deep blue bacterial colonies. By contrast, the control group was colorless.

Measurement of K_d Between MBP-YbaB and ClpY—QCM was applied by fixing MBP-YbaB on the sensor electrodes, and various volumes of ClpY protein were then loaded to measure the changes in the frequencies of the sensor cell. The average K_d of triplicate assays were $1.37 \times 10^{-9} \pm 3.12 \times 10^{-10}$ M. Fig. 3 showed one of the representative image of ClpY dose response curve. This K_d was smaller than the K_d (3×10^{-9} M) between ClpY and MBP-SulA that reported by Azim *et al.* (34). Therefore, the interaction between ClpY and MBP-YbaB was stronger than the interaction between ClpY and MBP-SulA.

MBP-YbaB In Vitro Degradation Test—After confirming the interactions between MBP-YbaB and ClpY proteins, we purified the ClpY, ClpQ, and YbaB proteins to investigate whether ClpYQ proteases can degrade YbaB. The His tag was used to purify the ClpY and ClpQ through a nickel column. The 6X His tag of ClpY was located at the N terminals, whereas that of ClpQ was located at the C terminals. MBP was fused to YbaB to purify the MBP-YbaB itself. Consequently, the molecular weight of the purified product was increased by ~ 40 kDa. The SDS-PAGE with Coomassie staining showed clear single band to ClpY, ClpQ, and YbaB (Fig. 4A).

The *in vitro* degradation test was configured by adding $2 \mu\text{M}$ ClpY, $2 \mu\text{M}$ ClpQ, $1 \mu\text{M}$ MBP-YbaB, and 5 mM ATP to the HEPES degradation buffer. The resulting solution was reacted at 37°C for 4 h, and samples were collected at 0, and 4 h after the reaction was initiated. The SDS-PAGE was used to observe the protein content. A reaction solution containing MBP-YbaB and ATP only was used as a control to determine whether the proteins can self-degrade within 4 h. The test results revealed the experimental group containing ClpYQ exhibited much higher degradation rate compared with that of the control without the proteases (Fig. 4B). Western blotting results also showed the degradation of YbaB after 4 h of reaction (Fig. 4C). This indicates that YbaB not only binds to ClpY but also is the substrate of ClpYQ.

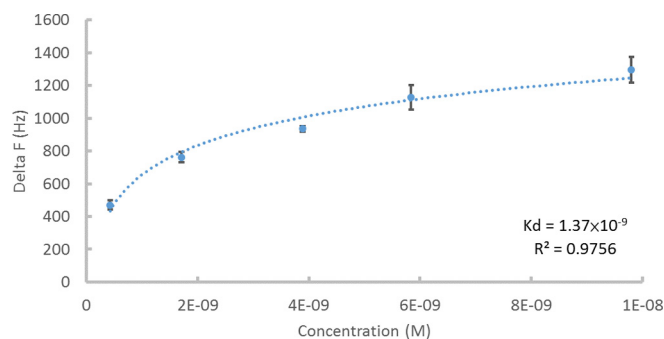


FIG. 3. **Determination of the K_d between ClpY and YbaB by QCM assay.** The assay was conducted by Affinix QN μ and performed in triplicate. The representative figure was chosen for showing curve fitting and K_d calculation.

MBP-YbaB In Vivo Degradation Test—In addition to exploring the *in vitro* degradation effect of ClpYQ, this study also applied *lon*⁻ *E. coli* strain (SG22623) and *lon*⁻ *clpQ*⁻ *clpY*⁻ *E. coli* strain (AC3112). The SG22623 was constructed from wild type, which was knocking out *lon* gene. The AC3112 was constructed from SG22623 by further knocking out the *clpQ* and *clpY* gene (35). These 2 KO strains will help us to investigate the *in vivo* degradation of YbaB. *E. coli* has two major ATP-dependent proteases, which are Lon and Clp proteases. Lon is responsible for the degradation of abnormal or unstable regulatory proteins in many bacteria. Moreover, the Lon protease shares same substrates with ClpYQ protease. It suggested the substrate of ClpYQ protease may be degraded by the Lon protease when *E. coli* suffering from ClpYQ functionless mutation (36). Thus, both strains SG22623 and AC3112 were considered as ATP-dependent protease deficient strains without the Lon protease, and the difference in the proteomic profiles between these two strains would be derived by the expression of ClpYQ protease in SG22623 (35). Samples of *ybaB* were cloned into pTH18kr-*mbp* plasmids and then transformed into SG22623 and AC3112 strains. IPTG was applied to express MBP-YbaB fusion proteins for 30 mins, and spectinomycin was then used to terminate the biosynthesis of the proteins in the bacterial cells. Therefore, the amounts of protein in the bacterial cells no longer increased. If the YbaB protein level decreased in SG22623 more than that in AC3112, this would confirm that YbaB proteins were degraded by ClpYQ *in vivo*. Western blotting with anti-MBP antibodies was used to measure the protein levels. The quan-

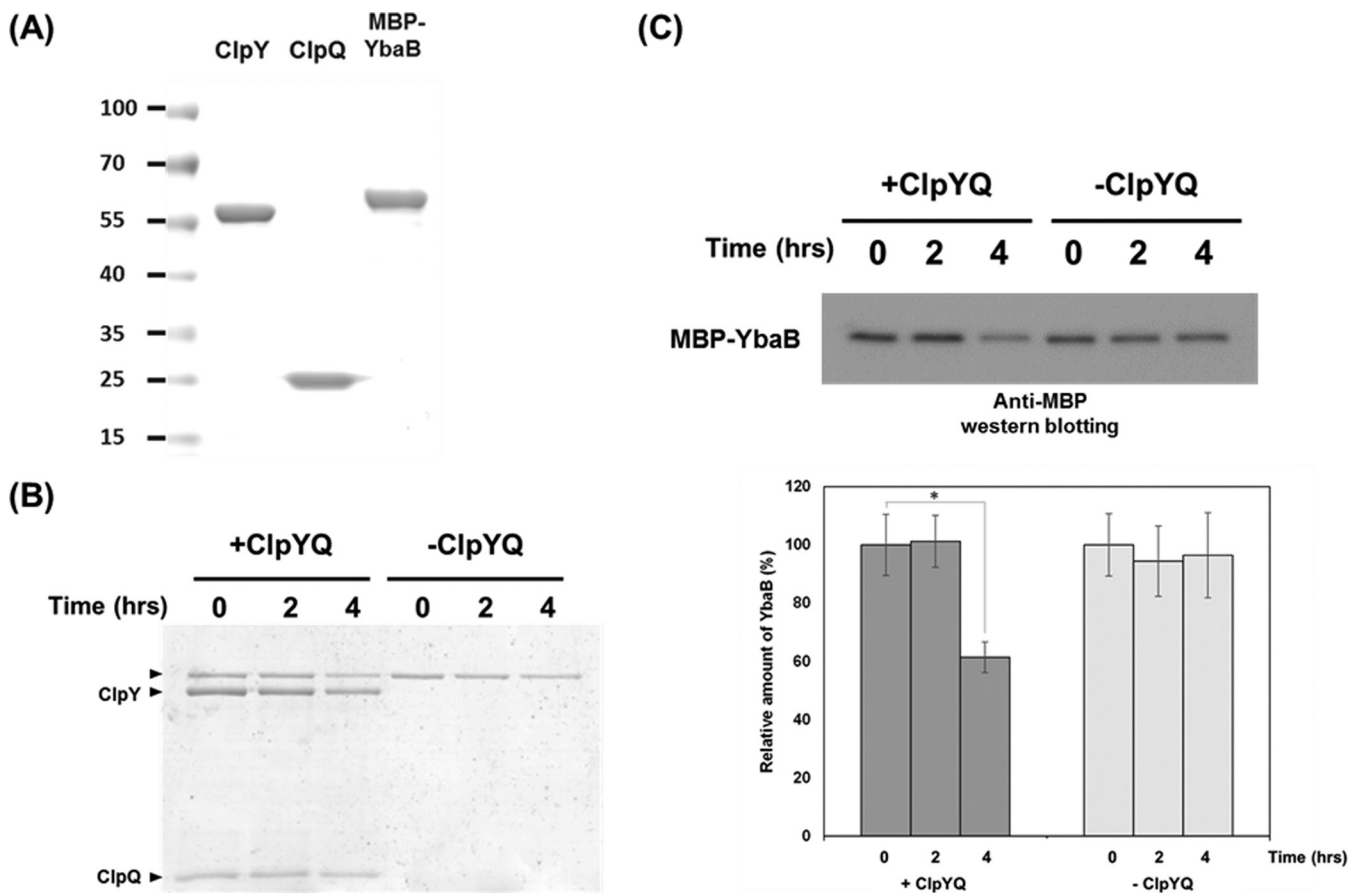


FIG. 4. *In vitro* degradation assay of MBP-YbaB. Degradations of MBP-YbaB by ClpYQ at 37 °C were determined by anti-MBP antibody and analyzed by Image J. A, Coomassie blue staining of purified MBP-YbaB, ClpY and ClpQ. B, SDS-PAGE of YbaB degradation by ClpYQ. (C) YbaB degradation by ClpYQ determined by Anti-MBP Western blotting. Means and standard deviations (error bars) were taken from three independent analyses. The asterisk indicates the significant difference by Student's *t* test ($p < 0.05$).

titative analysis results were standardized based on the bacterial growth OD_{600} values.

In Fig. 5, the protease deficient strain AC3112 shows no significant degradation of MBP-YbaB after 4 h. On the contrast, the Western blotting shows MBP-YbaB signal decreasing significantly after 4 h with ClpYQ coexpression strain SG22623 *in vivo*. This suggested the YbaB was degraded by ClpYQ proteolytic activity and the results are consistent with YbaB being a substrate of ClpYQ *in vitro*.

DISCUSSION

This is the first study to globally screen protease substrates using protein microarrays. Because the protein substrates on a protein chip would be degraded by the protease probe, it is very challenging to identify a protease substrate by using a regular proteome microarray assay directly. In this study, we chose an indirect strategy to first identify the protease-binding proteins. We used ClpY only, which is the recognition unit for ClpYQ protease, to screen for ClpY-binding proteins with *E. coli* proteome microarrays. After identifying the protein recognized by ClpY, we validated the protease activity by *in vitro*

and *in vivo* degradation assay. By using this indirect strategy, we identified that the YbaB is the substrate of ClpYQ.

When *E. coli* suffers in heat, the misfolding proteins will accumulate. Thus, the heat shock proteins are induced (37). However; in the normal physiological state, overexpression of heat shock proteins will be toxic to the cell. Some of antibiotics, such as puromycin, induced heat shock regulation protein expression. Missiaka *et al.* (19) founds ClpYQ is able to inhibit the constitutive heat shock regulation induced by puromycin. They reported the ClpYQ can degrade abnormal "puromycyl polypeptides". Thus, it is helping cell to lower the toxic effect of the abnormal heat shock response. The SOS response protein Sula (38) is a known substrate of ClpYQ (15). Sula inhibited the cell division when SOS response activated (38). The other knowing targets of ClpYQ are RcsA, RpoH, TraJ and RNaseR (15, 16, 39–41). RpoH is a heat shock transcription factor (16). RcsA is a capsule synthesis activation protein and also a substrate of Lon (42). TraJ is responsible for F plasmid conjugation activation (39). RNaseR is an important exoribonuclease responsible for degradation of structured RNAs in *E. coli* (41). These findings suggested the

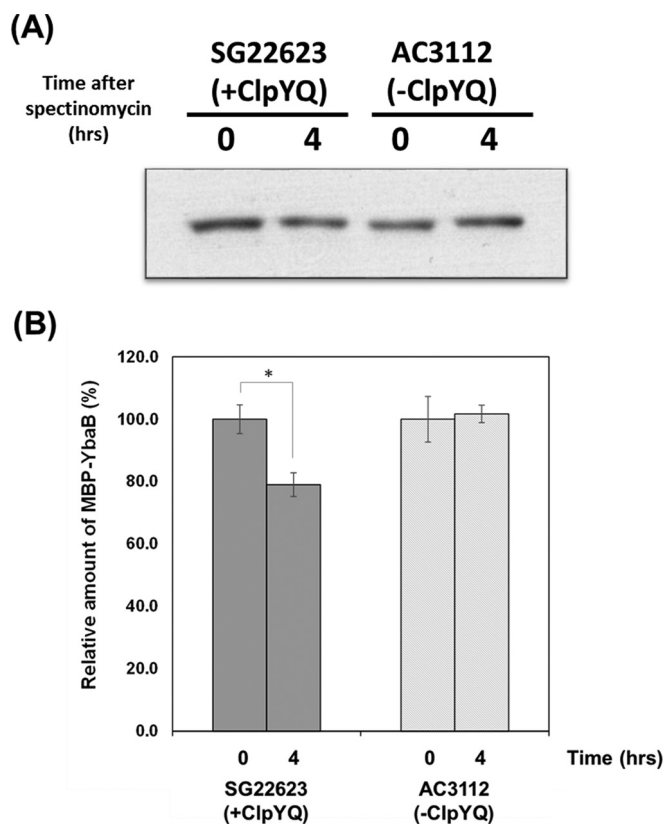


FIG. 5. *In vivo* degradation assay of MBP-YbaB. Determination of the degradation of MBP-YbaB in SG22623 and AC3112 by anti-MBP antibody. The bar chart was the quantification of degradation rate by Image J. Means and standard deviations (error bars) were taken from three independent analyses. The asterisk indicates the significant difference by Student's *t* test ($p < 0.05$).

ClpYQ participate in important cell physiological functions. In this study, we showed that YbaB is a novel target of ClpYQ. YbaB is a DNA binding protein with a probable histone-like activity in bacterial cells that facilitates packing the genetic materials of prokaryotes by forming nucleoids (43, 44). However, the actual function of YbaB remains unclear (43). Because YbaB is a histone-like protein, it may mediate gene-silencing and antisilencing activities through regulating the nucleoid structure (44). This suggested that ClpYQ protease participated in the YbaB's regulations functions and other gene regulation cascade. Also, YbaB shares homology with many pathogens, such as *Haemophilus influenzae*, *E. coli*, *Vibrio cholerae*, *Pseudomonas putida*, *Rickettsia rickettsiae*, *Neisseria gonorrhoeae*, *Bdellovibrio bacteriovorus*, *Clostridium perfringens*, *Bacillus subtilis*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Bacteroides capillosus*, and *Borrelia burgdorferi* (45). Their hosts include human, plants, and animals. This suggests that the regulation of YbaB function through digestion by ClpYQ is a very important mechanism in bacteria.

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