The degradation of RcsA by ClpYQ protease in *Escherichia coli*

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ABSTRACT

**Background:** RcsA is a positive activator that can transcribe capsular polysaccharide synthesis (*cps*) genes, and is degraded by the Lon protease. In *lon* mutant cells, the accumulation of RcsA leads to the overexpression of capsular polysaccharides. A previous study demonstrated that the overproduction of ClpYQ protease repressed the *cpsB::lacZ* expression, but no direct observation demonstrating that ClpYQ degrades RcsA has been reported.

**Results:** In this study, a newly created *malE-rcsA* fusion gene was genetically constructed in vivo, and its encoded fusion protein MBP-RcsA (maltose-binding protein fused with RcsA) was then purified. The in vivo measurement of the MBP-RcsA half-life was performed using SG22623 (*lon*) and AC3112 (*lon clpQ clpY*) strains. Comparative half-life experiments performed in the bacterial strains SG22623 and AC3112, demonstrated that the MBP-RcsA turnover rate in AC3112 was relatively slow and MBP-RcsA was stable at 30°C or 41°C. The in vivo and in vitro pull-down assays demonstrated that the ClpY was pulled down with MBP-RcsA. The rapid degradation of MBP-RcsA was observed in the presence of the ClpYQ protease at 41°C.

**Conclusion:** We concluded that RcsA was proteolized by ClpYQ protease.
The ATP-dependent protease ClpYQ is responsible for the degradation of abnormal proteins, along with Lon and ClpAP proteases in *Escherichia coli* [1-5]. ClpYQ is a heat-shock operon, which encodes ClpQ (19 kDa) (a small-subunit peptidase), and ClpY (49 kDa) (a large-subunit unfoldase) [6-8]. Each ClpQ or ClpY self-oligomerizes as a hexamer, and the four oligomers form a dumb-bell-shaped complex, \( Y_6 Q_6 Y_6 \) [9-12]. In the ClpYQ complex, ClpY transfers substrates from the external site of the cylinder into the catalytic core, where ClpQ degrades it with the expenditure of ATP(s) [9-12]. The ClpY contains three domains, namely N, I, and C [13-14]. The N-domain has an ATP-binding site with ATPase activity [9-12]. The I-domain protrudes outward to allow the gripping of substrates [15-17], and the C-terminal tail is necessary for the activation of ClpQ [18-19].

The ATP-bound hexameric ClpY is required for the recognition, unfolding, and translocation of the substrates [8]. Genetic and biochemical studies have revealed that the central pore I site of the ClpY was typically necessary to translocate the substrates [17, 20]. SulA, RpoH and TraJ, as well as a recent RNase R, have been identified as natural protein substrates [21-27]. The double-loop (aa 137 to 150 and 175 to 209) of ClpY I-domain is needed in vivo for the nascent recognition and binding of the natural substrates [28].

In *E. coli*, the RcsFCDB phosphorelay, a complex signal transduction system, was recently well-linked to functions involving the production of capsules, perturbation responses to cell envelope stress, the inhibition of biofilm formation, and the repression of swarming activities. [29-37]. Their genes encode RcsF (the outer membrane lipoprotein) [38-39], two inner membrane proteins, RcsC (the histidine kinase), RcsD (the histidine phosphotransfer, formerly called YojN) [31],
RcsB (the response regulator), and RcsA (the co-regulator) [30]. The role of RcsF in this signaling cascade is unclear. However, the cationic antimicrobial peptides (CAMPs) stimulate the Rcs-phosphorelay through the RcsF [40], and the RcsF serves as a ligand of RcsC and activates the Rcs signaling [41-42]. The autophosphorylated RcsC transfers the phosphate to the RcsD, and subsequently transfers the phosphoryl group to RcsB [35, 43]. Two classes of genes are activated by the RcsB; one is dependent on RcsA as co-activator (i.e., the \textit{cps} gene and \textit{rcsA} itself) [44-45] and the other is RcsA-independent genes, such as \textit{ftsA}, \textit{osmC}, \textit{rprA}, \textit{tviA}, \textit{ugd}, and \textit{gadA/BC}, notably through cooperation with other regulatory factors [46-52].

In addition, \textit{E. coli} becomes mucoid when its \textit{lon} gene has been deleted, because of an accumulation of RcsA [53-54]. RcsA and RcsB together bind to the RcsAB-box of the upstream of \textit{cps} genes (capsular polysaccharide synthesis) to activate its gene expression [55-57]. When ClpYQ proteases were overproduced in bacteria, the \textit{cpsB::lacZ} gene expression decreased, likely through an adjustment of RcsA levels [26]. However, in bacteria with an Alp\textsuperscript{+} [alternative Lon protease] phenotype, the RcsA was degraded, and the \textit{cpsB::lacZ} expression was low [58-60]. Unexpectedly, in an Alp\textsuperscript{+} strain lacking ClpY, the SulA accumulated. However, only the intermediate protein molecules, and no intact RcsA were observed [60]. Therefore, no direct evidence has been obtained that demonstrates that ClpYQ targets RcsA. Figure 1 shows the overall phosphorelay of the \textit{rcs} activation, and the likely proteolysis of RcsA by different proteases.

Because the RcsA easily aggregated in both the in vivo and in vitro analyses [55-57, 61], the MBP-RcsA fusion protein was made, and it was then used in both conditional analyses. The MBP-RcsA demonstrated normal activity for the activation of \textit{cpsB::lacZ} expression in the test. In addition, the over-production of
ClpYQ decreased the $cpsB::lacZ$ expression, most likely by targeting the MBP-RcsA. Both the MBP-RcsA and ClpY molecules were subjected to the in vivo pull-down assays. Additionally, the half-life of the MBP-RcsA was measured in the various protease-deficient strains. After the purification of the MBP-RcsA, the ClpQ and ClpY proteins, the in vitro pull-down analyses, and the degradation assays were each performed in the presence of ATP. Therefore, we concluded that the ClpYQ was involved in the RcsA degradation.
Methods

Bacterial strains and plasmids

The strains, phages and plasmids used in this study are listed in Table 1. E. coli. SG22623 (lon cpsB::lacZ) [62] and AC3112 (lon clpQY cpsB::lacZ) [26] both carry cpsB::lacZ fusion gene. The plasmid pTH18kr [63] was acquired from the National BioResource Project (NBRP) [64]. The plasmids pBAD33 and pBAD24 containing the pBAD promoter were under arabinose induction or glucose repression [65]. Plasmid pMAL-c2X was acquired from New England Biolabs (NEB), and the pET21a(+) from Novogene. The strains were cured of their kanamycin resistance cassette with pCP20, if required [66]. The P1 transduction was used to move ΔrcsA::kan or ΔrcsB::kan, into the MC4100 derivatives with the appropriate backgrounds [67].

Media and materials

The bacteria were grown in a Luria-Bertani (LB) broth supplemented with appropriate antibiotics or sugars. The supplements were added at the following concentrations when necessary: ampicillin (100 µg mL^{-1}); kanamycin (25 µg mL^{-1}); chloramphenicol (34 µg mL^{-1}); and 0.5% or 1% arabinose. The Taq DNA polymerase and restriction enzymes were purchased from Takara or NEB. Chemicals were obtained from Sigma or Waco.

PCR and plasmid constructions

To construct the plasmid pMALc2X-rcsA^{+}, the full-length rcsA^{+} gene was PCR(polymerase chain reaction)-amplified using primers EcoRI-rcsA-forward (5’-GCCGCGAATTCTAATGTCACCCGATTATTAGT-3’) and BamHI-rcsA-reverse (5’-GGCGGGATCCTTAGCAGTGTGACAAAAATACCA-3’), and MG1655
genomic DNA was used as the template. The DNA fragments from the PCR were then digested using EcoRI-BamHI, and cloned into the identical restriction sites of the pMAL-c2X. The resultant ligation mixture was transformed into XL-1 blue and selected on the LB-Amp\textsuperscript{r} plate. The transformant-carrying plasmids were isolated and characterized for the correct insert. To construct the plasmid pTH18kr-malE, the pMAL-c2X was digested using NdeI-HindIII, and the resulting DNA fragment carrying the malE gene and multiple cloning site (MCS) loci were ligated into the pTH18kr at the same sites. After the transformation and selection were completed, the correct resulting plasmid was designated as pTH18kr-malE. The plasmid pTH18kr-malE-rcsA\textsuperscript{+} was constructed by subcloning the EcoRI-BamHI restriction rcsA fragments of the pMAL-rcsA\textsuperscript{+}, into pTH18kr-malE at similar sites. A similar procedure was performed for the characterization of the plasmids with the right insertion. All the constructed plasmids with new pieces of DNA received from the PCR products were verified using DNA sequencing.

\textbf{β-galactosidase Assay of \textit{cpsB::lacZ} fusion gene expression}

\textit{E. coli} AC3112 (\textit{lon, clpQY, cpsB::lacZ}) cells carrying plasmids were grown overnight in LB with the appropriate antibiotic and arabinose. The overnight cultures were then 1:100 (v/v) sub-cultured in the same media. To induce the MBP-RcsA, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added in the exponential phase (\textit{OD}_{600} 0.3-0.4) to a final concentration of 1 mM for 2 h. Subsequently, the cells were collected and subjected to β-galactosidase assays. The β-galactosidase activities were assayed following the method described by Miller (1992). All the β-galactosidase activities were performed at least 3 times, and the values were determined in triplicate for each assay.

\textbf{In vivo pull-down assays of MBP-RcsA and ClpY}
Overnight bacterial cells, carrying pTH18kr-malE-rcsA<sup>+</sup> and pBAD24-clpY<sup>+</sup>, in LB with appropriate antibiotics, were 1:100 (v/v) and sub-cultured in the same media. After the cells were grown at 30°C in 25 mL of LB to an optical density at 600 nm of 0.3-0.4, the IPTG was added to a final concentration of 1 mM. After a 2 h induction of the MBP-RcsA, the cell cultures were collected using centrifugation. The samples were lysed with lysozyme (60 mg per mL) at 37°C for 1 h and then centrifuged. The cell lysates were supplemented with amylose resins (NEB) while being rocked overnight at 4°C. The mixtures were washed 10 times with a 50-mM HEPES buffer (pH 8.0) containing 300 mM of NaCl, 0.04% Triton X-100, 5% glycerol, and 5 mM of MgCl<sub>2</sub>. The proteins bound to the amylose resins were eluted with a 20-µL 2XSDS sampling buffer (0.75 mM of Tris-HCl (pH6.8), 7.5% SDS (sodium-dodecyl-sulfate) and 10% (v/v) 2-mercaptoethanol). The samples were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% slab gel, and detected using a western blot analysis with an anti-MalE antibody and anti-ClpY antibody.

**MBP-RcsA stability in various E. coli strains**

The measurement of the MBP-RcsA turnover rate was performed using the SG226223 (<i>lon</i>) and AC3112 (<i>lon</i>, <i>clpQ</i>, <i>clpY</i>) strains to compare the stability of the MBP-RcsA. Both strains carrying pTH18-kr-malE<sup>+</sup>-rcsA<sup>+</sup> were inoculated into 25 mL of fresh LB broth with appropriate antibiotics at 30°C or 41°C. The cultures were grown to the exponential phase (OD<sub>600</sub> 0.3-0.4), and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the 1-mM final concentration. To inhibit new protein synthesis, spectinomycin was added to a final concentration of 150 µg mL<sup>-1</sup> and the cell extracts were sampled at specific time intervals. The cell pellets were collected, washed twice, and re-suspended in a 2X SDS-PAGE sampling
buffer. After normalization, equal amounts of samples were loaded and electrophoresed on 12.5% SDS-PAGE. MBP-RcsA was then detected using an anti-MalE antibody. The western blots were developed using the ECL system and quantitated by Image J (version 1.45d) [68], and the turnover rate of the MBP-RcsA was determined.

**Protein expression and His-tagged purification of ClpY and ClpQ**

The ClpY was expressed with the N-terminal 6XHis-tagged using pET21a(+) in *E. coli.*BL21 (DE3), and the ClpQ was expressed using the C-terminal 6XHis-tagged. The cell cultures carrying plasmids were harvested and centrifuged after a minimum 6 h induction with 1 mM of IPTG at 30°C. The cell pellets were subsequently re-suspended in a native buffer (68 mM of NaCl, 58 mM of Na₂HPO₄, and 17 mM of NaH₂PO₄, pH 7.5) and lysed by sonication. After the cell lysate was centrifuged at 4°C, the supernatants were transferred into a TALON metal affinity resin column (Clontech) and flowed by gravity. The next sequential steps were: (1) Wash the resin by adding 10-20 bed volumes of wash buffer (50 mM of NaH₂PO₄, 300 mM of NaCl, pH 7.0); (2) Elute the 6XHis-tagged protein by adding elution buffer (50 mM of NaH₂PO₄, 300 mM of NaCl, and 150 mM of imidazole, pH 7.0), and collect the eluant. The protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as the standard [69], and the purified proteins were analyzed on an SDS-PAGE gel.

**Purification of the MBP-RcsA Fusion Protein**

*E. coli.*DH5α carrying pMalc2X-rcsA⁺ was inoculated into the LB with 0.2% glucose at 30°C. Because the cultures were grown with aeration to OD₆₀₀ of 0.4, the IPTG was added to a final concentration of 1 mM for a 6 h induction. After incubation,
the cells were harvested using centrifugation, and the supernatant was discarded. The
pellets were re-suspended with a buffer (20 mM of HEPES, 200 mM of NaCl, and 1
mM of EDTA, pH 7.4), sonicated, and centrifugation at 4°C followed. The
supernatant was loaded into amylose resin (NEB), and washed with 12 volumes of
column buffer. The fusion protein was eluted with elution buffer (20 mM of HEPES,
200 mM of NaCl, 10 mM of maltose, and 1 mM of EDTA, pH 7.4), and the
concentration was determined using Bradford protein assays [69], using BSA as the
standard.

**In vitro pull-down of MBP-RcsA and ClpY**

A pull-down of His-ClpY (6-monomer; 1.3 µM) in vitro was performed using
purified MBP-RcsA (7.8 µM) within a 50 to 150 mM-HEPES buffer (pH 8.0)
containing 150 mM of NaCl, 0.04% Triton X-100, 5% glycerol and 5 mM of MgCl₂.
After the MBP-RcsA and the His-ClpY were incubated at 37°C for 1 h, the samples
were mixed with 50 µL of amylose resins (NEB) while being rocked overnight at 4°C.
The mixtures were washed 10 times with 0.2 mL of 50-mM HEPES buffer (pH 8.0),
which contained 300 mM of NaCl, 0.04% Triton X-100, 5% glycerol, and 5 mM of
MgCl₂. Proteins pulled-down were eluted using 20 µL of 2X sampling buffer and the
resin-bound proteins were separated on 12.5% SDS-PAGE, followed by staining with
Coomassie blue R-250.

**In vitro degradation of MBP-RcsA by ClpYQ protease**

Reaction mixtures (60 µL) containing 2 µM of ClpY, 2 µM of ClpQ, and 1 µM
of MBP-RcsA in the 0.1-M HEPES buffer (pH 8.0), 10 mM of MgCl₂, 1 mM of
dithiothreitol (DTT), 1 mM of EDTA, and 5 mM of ATP were incubated for 4 h at
41°C and 30°C, respectively. After incubation, the reaction was ended by adding a
20-µL 2XSDS sampling buffer. The samples were subjected to 15% SDS-PAGE, followed by staining with Coomassie Blue R-250. The remaining percentage of MBP-RcsA was analyzed using Image J.
RESULTS

The MBP-RcsA activated the $cpsB::lacZ$ fusion gene expression with or without RcsA, and its activity decreased in the presence of ClpYQ Protease

Because the transcriptional activator RcsA easily aggregated [55-57, 61], the maltose-binding protein (malE gene) was added at its N-terminus to increase the solubility. The malE-$rcsA^+$ was constructed in a low copy plasmid pTH18kr, and the resulting plasmid was designated as pTH18kr-$malE-rcsA^+$. To examine the activity of the MBP-RcsA, the pTH18kr-$malE-rcsA^+$ was separately transformed into HT1001 ($lon$ $clpQ$ $clpY$ $rcsA$) and AC3112 ($lon$ $clpQ$ $clpY$), and both carried the $cpsB::lacZ$ fusion gene. After the IPTG induction, the samples of the two resultant transformants at different temperatures, were collected and subjected to β-galactosidase assays. Our results demonstrated that at 30°C, HT1001, in the absence of the Lon and ClpYQ proteases, as well as RcsA itself, with MBP-RcsA alone, had β-galactosidase levels of 400 units [Fig. 1(a)]. In contrast, 800 Miller units were obtained from AC3112 with MBP-RcsA. When the temperature was increased to 37°C or 41°C, the overall β-galactosidase levels of both strains decreased [Figs. 2(a) and 2(b)]. Because RcsA cooperated with RcsB to form a heterodimer to activate the $cps$ gene, to determine whether MBP-RcsA also worked with RcsB, an $rcsB$ deletion mutation was individually introduced into the AC3112 ($lon$, $clpQ$, $clpY$) and SG22623 ($lon$) mutants by P1vir transduction. The two resultant strains were separately designated HT1002 ($lon$, $rcsB$) and HT1003 ($lon$, $clpQ$, $clpY$, $rcsB$). After the transformation of pTH18kr-$malE-rcsA^+$ into these two strains, as compared with the parental SG22623 and AC3112, with pTH18kr-$malE-rcsA^+$, both transformants demonstrated decreased β-galactosidase levels [Fig. 2(c)]. These results indicated that MBP-RcsA cooperated with RcsB to activate the $cpsB::lacZ$ fusion gene. In addition, to demonstrate that
ClpYQ protease regulated the MBP-RcsA, the plasmids pBAD24-clpY+, and pBAD33-clpQ+ were transformed in series into the AC3112 strain-carrying pTH18kr malE-rcsA+. Using 1% arabinose to induce ClpQ and ClpY, the bacteria indicated lower β-galactosidase levels, compared with the control cells, carrying pBAD24 and pBAD33 together [Fig. 2(d)]. These results suggested that ClpYQ protease affected the stability of MBP-RcsA, and therefore inhibited the regulation of the cpsB::lacZ gene expression. To demonstrate that the MBP-RcsA was actually positively regulating the cpsB::lacZ expression, an anti-MalE antibody was used to detect MBP-RcsA in cells. The same samples were used in the β-galactosidase assays, and were subjected to western blotting analyses. As indicated in Fig. 2(e), the MBP-RcsA proteins were expressed well after the IPTG induction. However, the MBP-RcsA was less accumulated when the ClpYQ protease was overproduced [Fig. 2(e)], suggesting that the MBP-RcsA was likely targeted by the ClpYQ protease.

In vivo MBP-RcsA pull-down with ClpY

Our previous study and the experimental results of this study demonstrate that the ClpYQ protease regulated cpsB::lacZ indirectly by adjusting the RcsA levels. To verify that a direct interaction between ClpY and RcsA occurred, the AC3112 strain was transformed using pBAD24-clpY+ and pTH18kr-malE-rcsA+ plasmids or the control plasmid pBAD24-clpY+ and the two resultant co-transformant cells were used for the pull-down assays. The control cells only expressed ClpY, and the experimental cells expressed a pair of protein molecules (ClpY/MBP-RcsA). The bound ClpY molecules were precipitated with MBP-RcsA in amylose resin mixtures. The MBP-RcsA and ClpY molecules were both detected in western blot analyses using
specific antibodies (Fig. 3). In contrast, in bacterial cells without MBP-RcsA, ClpY protein was not detected by the anti-ClpY antibody (Fig. 3). These results indicated that the RcsA in the cells had marked affinity for ClpY.

**In vivo MBP-RcsA proteolyzed by ClpYQ at higher temperature, independent of RcsB**

To confirm that the induced MBP-RcsA was regulated by the chromosomal ClpYQ protease, SG22623 (lon) and AC3112 (lon clpQ clpY) strains were used to measure its half-life in two different strains. Again, the pTH18kr-malE-rcsA+ was transformed into the SG22623 (lon) and AC3112 (lon clpQ clpY) strains, and the resultant transformants were grown at 30°C and 41°C. After adding spectinomycin at the log-specific time intervals, the cell cultures were collected and subjected to western blot analyses with an anti-MalE antibody. At 30°C, the MBP-RcsA protein in the lon clpQ clpY (AC3112) triple mutant was quite stable (Fig. 4A, right panel). By contrast, in the lon deletion mutant (SG22623), the decay of MBP-RcsA subtly increased (Fig. 4A, left panel). After the Image J analysis was conducted, the half-life of the MBP-RcsA was approximately 1.5 h longer than in SG22623 (Fig. 4D, left panel). At 41°C, the amount of MBP-RcsA in the SG22623 (lon) strain gradually decreased (Fig. 4B, left panel). The half-life of the MBP-RcsA was approximately 30 min (Fig. 4D, middle panel). In contrast, the MBP-RcsA were stable in AC3112 (lon, clpQ, clpY) (Fig. 4B, right panel), and the turnover rate was relatively slow (Fig. 4D, middle panel). We also performed a half-life measurement of MBP-RcsA in HT1002 (lon, rcsB) and HT1003 (lon, clpQ, clpY, rcsB), and both strains carried pTH18kr-malE-rcsA+. The decay of the MBP-RcsA was measured in the two transformants. Similarly, the degradation of MBP-RcsA was faster (Fig. 4C, left panel) and its half-life was approximately 30 min in HT1002 (lon, rcsB) mutants at 41°C.
By contrast, the MBP-RcsA was relatively stable in HT1003 (lon, clpQ, clpY, rcsB) (Fig. 4C, right panel). The half-life of MBP-RcsA was increased 5-fold for approximately 150 min (Fig. 4D, right panel). The overall results demonstrated that the ClpYQ degraded the MBP-RcsA at a higher temperature with or without RcsB.

**In vitro pull-down of His-ClpY and MBP-RcsA**

To further verify the interaction of ClpY with MBP-RcsA, the ClpY was also pulled-down with MBP-RcsA in vitro as determined by two separate plasmids (pET-his-clpY+ and pMalc2X-rcsA+) that were used for protein purification. The ClpY with the N-terminal (His)6-tag was purified using the His-tag affinity methods, and the MBP-RcsA fusion protein was purified using amylose resin. The purified proteins are illustrated in Fig. 5(a). An equal molar of His-ClpY and MBP-RcsA were mixed with resin. After 4 h of incubation, the precipitated proteins were dissolved in the sample loading buffer during the subsequent centrifugation. As demonstrated in the SDS-PAGE gel, the His-ClpY associated with MBP-RcsA, and both protein molecules were detected [Fig 5(b)]. However, without the addition of MBP-RcsA, the His-ClpY was not detected [Fig 5(b)]. Similar results were obtained in western blot analyses in which both the His-ClpY and RcsA molecules were detected [Fig 5(c)].

**In vitro degradation of MBP-RcsA at higher temperatures**

To highlight that the ClpYQ protease degraded the MBP-RcsA, the purified ClpQ with C-terminal (His)6-tag and ClpY with N-terminal (His)6-tag, as well as the MBP-RcsA were added together in vitro in the presence of ATP. The 2:2:1-μM concentration for His-ClpY(6x), His-ClpQ(6x), and MBP-RcsA were mixed during the degradation reaction at 30°C or 41°C for 4 h. Consequently, the MBP-RcsA was
markedly degraded at 41°C (Fig. 6). The Image J program was used to analyze the amount of MBP-RcsA, and the relative levels were calculated and plotted with the time intervals. As indicated in Fig. 6, after 4 h of incubation, 90% ± 2% of the MBP-RcsA remained at 30°C, and 79% ± 2% of the MBP-RcsA remained at 41°C.
DISCUSSION

In this study, the MBP-RcsA activated the \( \text{cpsB::lacZ} \) fusion gene expression. However, in the absence of RcsB, similar to the RcsA, the MBP-RcsA did not activate the \( \text{cpsB::lacZ} \) gene expression. Because the MBP-RcsA highly activated the \( \text{cpsB::lacZ} \) in the presence of the chromosomal RcsA, the MBP-RcsA/RcsA with RcsB most likely formed the heterodimers that activated the \( \text{cpsB::lacZ} \) gene expression. However, because the ClpYQ protease was overproduced in cells, both the \( \text{cpsB::lacZ} \) expressions and MBP-RcsA levels decreased (Fig. 2). Therefore, the ClpYQ regulated the \( \text{cpsB::lacZ} \) by adjusting the MBP-RcsA/RcsA levels.

Through the co-expression of the MBP-RcsA and ClpY, the ClpY and MBP-RcsA were pulled-down together. However, in the presence of the RcsB at 30°C, the MBP-RcsA demonstrated stable levels in bacterial cells. Nevertheless, the MBP-RcsA had a slightly longer half-life in the absence of both Lon and ClpYQ proteases. Because the RcsB with RcsA formed the heterodimers that were bound to the RcsAB box, the RcsB appeared to protect the MBP-RcsA from the proteolysis by ClpYQ proteases. At 41°C, the MBP-RcsA had a shorter half-life in the lon mutants, but had a much longer half-life in the \( \text{lon clpQ clpY} \) mutants. Next, the amount of ClpQ and ClpY was once again increased, at elevated temperatures to achieve faster degradation. Nevertheless, without the RcsB, the MBP-RcsA demonstrated a similar half-life as did the rcsB\(^{+}\) cells at 41°C. Therefore, at an elevated temperature, the RcsB was not likely to be associated with the MBP-RcsA. All the observations were reproducible by growing the bacteria at 37°C (data not shown). To support this, in the previous in vitro analyses, the mixture of the RcsA and RcsB bound less to the RcsAB box in the DNA fragments, at 37°C [55]. Under these conditions, the RcsA and RcsB did not display similar functionality at higher temperatures. These reports, as well as previous reports have demonstrated that the RcsB was capable of being
associated with other factors to bind the various DNA promoter sites in the RcsA-independent mode. Therefore, as the temperature increased, without the RcsB association, the RcsA itself was not only defective for the capsular synthesis, but also had a distinct role compared to the RcsB. Notably, the decay of the MBP-RcsA leveled-off in bacteria that lacked both the ClpYQ and Lon proteases [Fig. 4(d), middle panel]. Therefore, the ClpYQ protease was mostly responsible for the degradation of the MBP-RcsA. However, without RcsB, the decay of the MBP-RcsA trailed-off [Fig. 4(d), right panel]. It is likely that the other proteases may also be involved in the degradation of MBP-RcsA.

In this study, we demonstrated that RcsA is the substrate of ClpYQ protease. However, it has also been demonstrated that under stress conditions, the RcsA was not accumulated in the Alp$^+$ (alternative Lon protease) strain [60]. Because it was also devoid of the ClpY protease, no RcsA was detected, and only fragmented-RcsA accumulated [60]. Therefore, the RcsA either self-cleaved or was proteolyzed by other proteases. In this study, in the absence of RcsB, the MBP-RcsA was not stable in the \textit{lon} and \textit{lon}, \textit{clpQ}, \textit{clpY} mutants at 30°C (data not shown). Therefore, other proteases were also involved in the proteolysis of MBP-RcsA. Future studies should determine whether other proteases are also involved in Alp$^+$ degradations.

Conclusion

Direct evidence of ClpYQ protease targeting the RcsA was not observed. However, the ClpYQ protease in \textit{E. coli} proteolized the RcsA, and highlighted that other proteases were likely responsible for the alternative Lon protease function.

Competing interests

The authors declare that they have no competing interests.
Authors’ contributions

HTH performed the β-galactosidase assays, western blot analyses, pull-down analyses.

HTH and FCH performed the degradation assays. All the authors participated in the study design and data analysis.
ACKNOWLEDGMENTS

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Table 1. Bacterial strains, phages and plasmids used in the study

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<tr>
<td>DH5α</td>
<td>F- φ80dlacZΔM15</td>
<td>Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK^- mK^+) phoA supE44 λ^- thi-1 gyrA96 relA1</td>
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<tr>
<td><strong>Phage</strong></td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pTH18kr</td>
<td>Kan¹; ori(pSC101); Ptac promoter</td>
<td>[63]</td>
</tr>
<tr>
<td>pBAD24</td>
<td>ori(pBR322); araC P_{BAD}; Amp^f</td>
<td>[65]</td>
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<tr>
<td>pBAD33</td>
<td>ori(pACYC); araC P_{BAD}; Chl^f</td>
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<tr>
<td>pET21a</td>
<td>Amp^f; T7 promoter</td>
<td>Novagen</td>
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<tr>
<td>pMAL c2X</td>
<td>ori(pBR322) Ptac Amp^f</td>
<td>New England Biolabs</td>
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<td>pBAD24-clpY^+</td>
<td>clpY^+ in pBAD24</td>
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<td>pBAD33-clpQ^+</td>
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<tr>
<td>pTH18kr malE-rcsA^+</td>
<td>malE-rcsA^+ fusion gene under Ptac promoter</td>
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<td>6×His-tagged clpQ^+ in pET21a</td>
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<td>pET21a-(6×His)-clpY^+</td>
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<td>pMALc2X-rcsA^+</td>
<td>rcsA^+ in pMAL-c2X</td>
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<td>pCP20</td>
<td>FLP^+ cI1857^+ P_{R Rep^a} Amp^f</td>
<td>Chl^f</td>
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Figure Legend:

Figure 1. The Rcs phosphorelay of rcs regulation and proteolysis of RcsA in *E. coli*.

The RcsF receives signal and activates the kinase activity of sensor kinase RcsC. It is unknown whether it is direct or indirect. Indirect activation is likely via IgaA(YrfF) since it can repress the kinase activity of RcsC [29]. The phosphorylation of RcsB is mediated by the RcsC and RcsD via a complex phosphorelay and vice versa. The phosphor-RcsB regulating some genes is RcsA-dependent. The expression of the *cps* operon and the repression of *flhDC* operon is RcsA-dependent, whilst the regulation of *ftsZ*, *rprA* and *osmC* expression is RcsA-independent. RcsA, an unstable protein that is degraded by Lon and likely by other ClpYQ and Alp⁺.

Figure 2. Activity of MBP-RcsA monitored via chromosomal *cpsB::lacZ* fusion gene, and the overproduction of ClpYQ protease affect expression of *cpsB::lacZ*. The β-galactosidase levels of strains that carry pTH18kr MBP-rcsA with *cpsB::lacZ* cultured at different temperature (30°C, 37°C and 41°C) after induction IPTG and the units were calculated at least in three times. (A) HT1001 (*lon, clpQY, ΔrcsA*) (B) AC3112 (*lon, clpQY*) (C) SG22623 (*lon*) and AC3112 (*lon, clpQY*) strain in the presence of *rcsB*, the isogenic HT1002 (SG22623ΔrcsB) and HT1003 (AC3112ΔrcsB) strain in the absence of *rcsB* (D) AC3112 carrying pBAD24-*clpY*, pBAD33-*clpQ* and pTH18kr MBP-rcsA were grown at 30°C in LB with 1% arabinose and induced by IPTG for production of MBP-RcsA. Expression of MBP-RcsA proteins and β-galactosidase activity were measured from the same sample. (E) MBP-RcsA levels were detected by Western blot with anti-MalE antibody.

Figure 3. *In vivo* ClpY was pulled-down with MBP-RcsA. AC3112 strains carrying pBAD24-*clpY*/pTH18kr MBP-rcsA or pBAD24-*clpY* were grown at 30°C and induced for the production of ClpY and MBP-RcsA. The left lane, cells without MBP-RcsA, the right lane cells with MBP-RcsA and ClpY. The proteins bound to resins were subjected to the immunoblotted analyses, using anti-ClpY and anti-MalE antibody.

Figure 4. *In vivo* turnover rates of MBP-RcsA. MBP-RcsA turnover was measured in cultures of *lon* and *lon clpY clpQ* strains grown at 30°C or 41°C separately. After addition of spectinomycin, the relative change in the amount of MBP-RcsA was measured by immunoblotting with anti-MalE.
antibody. (A) SG22623 (lon mutant) and AC3112 (lon clpY clpQ mutant) were grown at 30°C. (B) SG22623 and AC3112 were grown at 41°C. (C) HT1002 (lon rcsB mutant) and HT1003 (lon clpY clpQ rcsB mutant) were grown at 41°C. (D) The relative change in band intensity of MBP-RcsA was analyzed by Image J.

Figure 5. Proteins purification and ClpY interacting with MBP-RcsA in vitro. (A) The ClpY with the (His)$_6$-tag were purified using His-tag affinity, while MBP-RcsA were purified by amylose resin methods. The purified proteins were subjected to 15% SDS-PAGE. (B) His-ClpY (49kD) has affinity toward MBP-RcsA (63 kD) and was pulled down by amylose resin. (C) Detection of the interactions between ClpY and MBP-RcsA through Western blot.

Figure 6. MBP-RcsA in vitro degradation by the ClpYQ protease. (A) The molar ratio 2 : 2 : 1 µM of His-ClpY His-ClpQ and MBP-RcsA were used at 30°C or 41°C. At time intervals, 0, 2, 4 hrs, the samples were extracted and were subjected to SDS-PAGE on a 15% gel and stained with Coomassie blue R-250. (B) The relative change in band intensity of MBP-RcsA at different time points was analyzed by Image J.
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Figure 2

A

![Graph A](image_url)

B

![Graph B](image_url)

C

![Graph C](image_url)

D

![Graph D](image_url)

E

![Graph E](image_url)
Figure 3