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Targeted protein degradation is a powerful tool that can be used to create unique physiologies depleted of important factors. Current strategies involve modifying a gene of interest such that a degradation peptide is added to an expressed target protein and then conditionally activating proteolysis, either by expressing adapters, unmasking cryptic recognition determinants, or regulating protease affinities using small molecules. For each target, substantial optimization may be required to achieve a practical depletion, in that the target remains present at a normal level prior to induction and is then rapidly depleted to levels low enough to manifest a physiological response. Here, we describe a simplified targeted degradation system that rapidly depletes targets and that can be applied to a wide variety of proteins without optimizing target protease affinities. The depletion of the target is rapid enough that a primary physiological response manifests that is related to the function of the target. Using ribosomal protein S1 as an example, we show that the rapid depletion of this essential translation factor invokes concomitant changes to the levels of several mRNAs, even before appreciable cell division has occurred.

Traditional approaches to unravel protein-coding gene function include making knockout or conditional mutant strains for comparative studies. In many cases, obtaining a conditional inactivation mutant is not tractable or there may be concerns that the inactivated protein is defective only in one of several traits. When investigating essential genes, knockout strains can be cultured for biochemical analyses when there is a complementing copy of the gene. By placing an essential gene under the control of a regulated promoter, so-called depletion-by-division experiments can be performed wherein the gene is turned off, and then cell division and normal protein turnover are used to deplete the encoded factor from the cell. Complications arise when the depletion of a factor induces downstream physiological responses that are not directly related to the factor's function, one example being the activation of multiple toxins when translation is inhibited (12, 52, 56). A solution to this problem is to specifically target the encoded gene product for conditional depletion (29). In doing so, the gene remains in its natural context, and the encoded product functions normally at the appropriate dose prior to its removal.

Prior studies have demonstrated the utility of this approach for specific cases by reengineering target proteins such that they contain a peptide degradation signal that is recognized by a processive protease (7, 15, 20, 24, 29, 53). In early examples, a degradation peptide that has a weakened affinity for the protease was selected, and then an adapter protein that increases the effective local concentration of the target near the protease to increase degradation was conditionally expressed (20, 29). In another example, a cryptic degradation signal was used that was liberated by the conditional expression of a second, site-specific protease (53). In each case, the degradation efficiency of the system for any given target protein is unpredictably variable, so substantial time and effort may be required to engineer the system to achieve a suitable balance between "off" and "on," such that essential targets are not appreciably depleted prior to the intended experiment when the system is off and that they are thoroughly depleted when the system is on. Moreover, the time required for depletion of the target after activating the degradation system may be quite long, which obfuscates the resulting experimental data.

The conditional depletion of specific proteins provides access to unique cell physiologies that can be telling of the protein's function. We reasoned that if a target protein could be very rapidly depleted, then a "cell reflex" should be observable that is uniquely related to the disturbed pathway. Such a reflex would include rapid changes to mRNA levels and metabolites that are somehow related to the function of the target protein. Our search for a rapid degradation system that worked on a wide variety of protein targets without substantial optimization led us to the development of a simple degradation system in which the target protein is modified by the addition of a degradation tag and the degradation protease is conditionally expressed. Here, we demonstrate that our degradation tag is tolerated on a wide variety of important proteins. Moreover, using ribosomal protein S1 as an example, we demonstrate a cell reflex: upon its rapid depletion, changes in the levels of several mRNAs were observed that were concomitant with the removal of the target. Interestingly, we discovered that the levels of mRNAs encoding GAPDH and CsdA were markedly influenced by S1.

MATERIALS AND METHODS

Bacterial strains and plasmids. Recombineering to add the control and degradation tags was performed in strain SM1405 (X90 Δ*clpX* Δ*clpA* Δ*rna*, with pSIM-5) (14, 22). These loci were then transduced with phage P1 by selecting for the adjacent drug resistance markers into G78 (BW30270, an *fnr*$^+$ *rph*$^+$ derivative of MG1655, with both the *clpX* and *rna* open reading frames [ORFs] replaced by FLP recombination target [FRT] sites, and the *araBA* ORFs replaced with a chloramphenicol resis-

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tance marker) (13, 32). The growth studies were performed using LB supplemented with 0.2% glycerol and either 0.2% glucose or 0.2% arabinose as indicated. The ClpXP expression library was constructed by first amplifying *clpP* and *clpX* from genomic DNA with primers that randomized two nucleotides in their respective Shine-Dalgarno sites and then using PCR to fuse the genes in their natural order (45). The fusion product was digested with restriction sites encoded by the primers and then ligated under the control of the P_{BAD} promoter in a pBR322-based plasmid containing *araC*. The pClpPX plasmids were maintained with 125 μg/ml of ampicillin.

Recombining the control and degradation tags onto target genes. The control and degradation tag sequences were constructed using PCR and fused using PCR to a downstream antibiotic resistance marker (either the 6′-aminoglycoside acetyltransferase [Kan^r] ORF lacking a promoter, or the Tet^r gene from pBR322 containing its own promoter) such that they contained 50 base pairs of homology to the regions flanking the stop codons of the target genes. The products were electroporated into recombinogenic SM1405 and selected on the appropriate antibiotic (14). Resistant colonies were restreaked under selection, and the resulting colonies were screened using diagnostic PCRs that amplified the region surrounding the stop codon of the target gene. The addition of the degradation tag and the adjacent resistance marker substantially increased the size of the resulting PCR product and made concomitant detection of wild-type and modified target genes possible. These products were gel purified and sequenced to confirm the tag integrity. Phage P1 was used to transduce the tagged genes using selection for the adjacent markers into strain G78. The tags in the resulting transductants were resequenced.

Selection of tailored protease expression plasmids. Strains with degradation tags on established essential genes were transformed with the pClpPX library and selected on LB-ampicillin-glucose plates. Transformants were picked with toothpicks and lightly patched onto plates with glucose or arabinose and incubated ~16 h at 37°C. Patches that showed sickness on arabinose (overall slow growth with dispersed fast-growing lobes) were used to identify candidate clones for degradation studies. The pClpPX plasmids were purified from the parallel glucose patch and used to transform both the target control and target degradation strains, selected with glucose, and then grown in LB-glucose until late-exponential phase. These cultures were serially diluted in ice-cold LB, and spots were plated on both glucose and arabinose to assess the influence of ClpXP expression. After overnight incubation, strains were identified that grew well on glucose and poorly on arabinose. These were then stocked as paired control and degradation strains, with care taken to prevent extended starvation periods, which increase the abundance of cells that are refractory to degradation.

Growth analysis. Overnight cultures of test strains were diluted 1:100 in fresh medium, and 3 to 5 samples were aliquoted into the central wells of a 96-well plate. The outer wells were filled with water, and the plates were incubated at 37°C with continuous shaking in a Biotek Synergy MX plate reader. Culture turbidity was measured every 5 min, and the resulting data from each well was averaged. For manual inductions, the plate reader was paused, sugars were added, and then measurements were resumed within 1 min. Growth rates were determined by transforming the raw data into $\log_2$ and using the derivative to establish the steepest log-linear range. Linear regression was then used on that region of the $\log_2$ data, and the doubling time was calculated from the inverse slope (31). Depending on the experiment, different measured wavelengths and culture volumes (50 to 100 μl) account for differing final turbidity readings.

Target degradation and sample preparation. Overnight cultures of the control and degradation strains were diluted 1:100 into ampicillin-glycerol medium and grown at 37°C with aeration. When the cultures were in exponential-phase growth, uninduced samples were withdrawn and then 0.2% arabinose was added to induce ClpXP expression. At 10-min intervals, individual samples were taken for turbidity measurements, for total protein recovery, and for RNA isolation. The samples for protein analysis were mixed with ice-cold protease-inhibitor cocktail (Roche) prior to harvesting at 4°C, and the cells were then lysed in B-Per2 (Pierce) supplemented with 1 mM EDTA and 0.1 mg/ml lysozyme. After lysis, the samples were supplemented with an equal volume of a mixture containing 25 mM K-HEPES, 5 mM $MgCl_2$, and 10 μl/ml Benzonase (Sigma) and incubated an additional 5 min. Volumes were adjusted to normalize each sample according to the turbidity of the cultures when harvested, and an aliquot of each was mixed with SDS sample buffer and heated prior to electrophoresis. Total RNA was prepared following slight modifications to established protocols (5, 25, 55), quantified spectrophotometrically, and adjusted to 1 mg/ml in 10 mM bis-Tris, 0.1 mM EDTA, pH 6.5, prior to storage at −80°C.

mRNA quantification. A commercial random priming kit was used to prepare cDNA from 0.5 μg of each RNA sample (iScript; Bio-Rad), and then each library was diluted 4-fold in water and stored at −80°C. Quantitative PCR was performed using primers that amplified ~90- to 120-bp segments from the extreme 5′ ends of each gene's ORF with a commercial mixture of buffer, fluorescent dye, and polymerase (SsoFast; Bio-Rad). Amplification was carried out and recorded using a Bio-Rad MiniOpticon detection system. Rare transcripts were quantified without further dilution of the cDNAs, and abundant transcripts were quantified following an additional 20-fold dilution. The raw data from the amplifications were analyzed in spreadsheets after baseline optimizations to assign cycle thresholds with a higher degree of accuracy than the onboard software prior to averaging replicates (42). The high-low boundaries were assigned for each data point by combining three measurements: (i) the variance in the measurement of a single transcript following six independent quantitative PCR (qPCR) measurements from the same cDNA sample (which established error from mixing), (ii) the difference between calculated abundance using the in-well amplification efficiency versus the averaged efficiency for each target (which accounts for deviance in slope calculations), and (iii) the difference in apparent relative abundance to uninduced samples when the experimental cycle thresholds were separately compared against the basal levels obtained from different cultures. The latter measurement also allowed for an error estimate of the amount of basal transcript present in the uninduced samples (to which all others in a series were compared) and accounted for the most of the observed variances.

RESULTS

Design of a broadly applicable protein degradation system. We had an interest in rapidly degrading a wide variety of essential and nonessential proteins using a targeted degradation system. In preliminary work, we had variable success using an established degradation system that relies on using the SspB adapter to deliver substrates to ClpXP (20). One complication that may have affected the degradation efficiency in some cases is the fact that SspB is a dimer, so its expression can lead to cross-linking of multimeric targets and impede delivery to ClpXP. Normally, this is not a problem for SspB-mediated delivery of tmRNA-tagged proteins, because the tags are present only on a small fraction of a given protein pool (41).

We reapproached targeted degradation with a less-elegant strategy: by modifying all targets with a common degradation tag and regulating the activity of ClpXP, we were able to conditionally degrade a broader range of cytosolic targets (Fig. 1A). The degradation peptide tag was designed that combines the ClpX recognition element of the *ssrA* peptide from *Escherichia coli* with epitopes that can be used to track and purify the intact target protein (18). This tag also contains flexible linkers and positions the ClpX recognition determinant sufficiently far from the target body to allow for efficient engagement by ClpXP (Fig. 1B) (28, 34). Processive unfolding and degradation of the target by ClpXP results in the disappearance of the FLAG epitope, because the distance from the

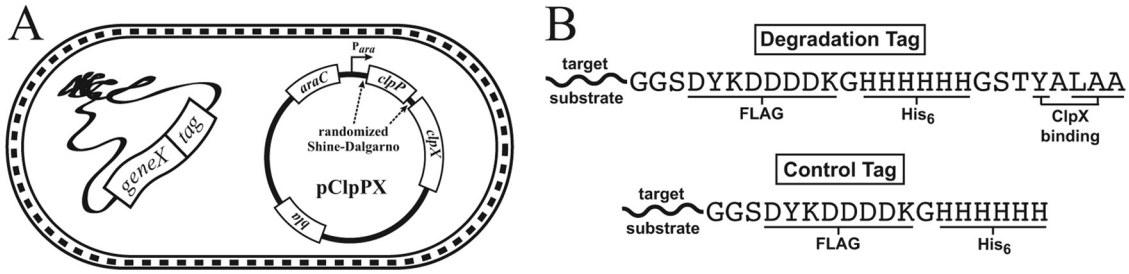

FIG 1 A controllable protein degradation system and peptide tags. (A) The chromosomal version of the target gene is modified in its normal locus to encode a peptide tag on the C terminus of the protein (geneX tag). ClpP and ClpX are expressed from an arabinose-inducible plasmid (pClpPX). To match proteolysis activity with target abundance and stability, variants of the plasmid are selected containing altered translation initiation sequences for each protease component. (B) Schematic of the degradation tag containing a FLAG and His_6 sequence as well as a C-terminal ClpX recognition motif. The control tag lacks the ClpX recognition determinants.

entrance of ClpX to the proteolytic lumen of ClpP is approximately 40 amino acids in length (28, 34). In addition, a stable variant lacking the ClpX recognition sequence was also designed for use in parallel control strains so that the effects of target degradation could be parsed from any unrelated effects stemming from ClpXP expression.

The sequence encoding these tags and an adjacent antibiotic resistance marker were amplified by PCR using primers that added homology arms to the regions flanking the stop codon of each target. Recombineering was then used to introduce the tag sequences onto the 3′ end of the target gene open reading frame in Δ*clpX* cells such that the tagged versions of the target genes were the only copies present (14). After recombination, diagnostic PCRs and DNA sequencing were used to confirm the addition of the tag sequence and to verify that the unmodified, wild-type genes were no longer present. The tagged versions of the target genes were then transduced using P1 phage into naive, Δ*clpX* cells prior to growth and degradation studies.

Diverse proteins bearing the control and degradation tags. Unlike the natural *ssrA* peptide, the reengineered degradation tag was expected to maintain target protein stability in the absence of ClpX because it did not contain the ClpA or SspB binding sequences, which, respectively, promote ClpAP-mediated degradation and target dimerization (6, 18, 27). To evaluate the general tolerance of the degradation and control peptides, we appended them to a variety of important proteins (Table 1). For each of these cases, we were able to generate strains harboring only the tagged versions of the target genes. Moreover, the growth of these strains in liquid culture was generally robust (Fig. 2). Thus, the addition of these peptide tags did not substantially destabilize the target proteins.

Because the degradation system can be used to monitor cell physiology upon target depletion in large cultures, it was important to establish the genetic stability of the tag sequences after extensive passaging. Therefore, we resequenced the tagged target genes after serial culturing. In all cases but one, the original tag sequences were maintained. The exception was the version of *ftsZ* encoding the degradation tag, which repeatedly acquired frameshift mutations. The control-tagged version of this gene was stable.

Our high success rate in appending tags to the C termini of essential proteins was anticipated, because many *E. coli* proteins tolerate C-terminal fusions, and important surface features of proteins generally do not include either terminus. Nonetheless, we wanted to establish the consequence of attempting to add these tags to an essential protein that should not tolerate a C-terminal modification to see if we could distinguish simple recombineering failures from essential pathway interruptions. For this purpose, we targeted *rplP* (encoding ribosomal protein L16) because it is essential, and L16's C-terminal methionine and carboxylate form intimate contacts with the neighboring L25 in the crystal structure of the *E. coli* ribosome (4). Surprisingly, we were able to recombine both the control and degradation tags onto the *rplP* gene, but these strains maintained a second, wild-type copy that was readily detectable using diagnostic PCR. Therefore, in cases where the addition of the tag sequences interferes with an important function, selective pressures can promote the accumulation of mutated tags or merodiploidy.

Controllable target degradation. An inability to transduce target genes bearing degradation tags (target-deg) into $clpX^+$ $clpP^+$ cells is a strong indication that the target is essential and degraded well enough by endogenous ClpXP to inhibit colony formation. However, in some cases, we found discrepancies between anticipated transduction efficiencies and the reported essentiality of target genes bearing degradation tags. We further investigated two cases that yielded ambiguous data. In one, *frr*-deg was able to be transduced into $clpX^+$ cells, although at a consis-

TABLE 1 A collection of genes tagged with control and degradation tags[a]

Target gene	Protein function	Essential?	Degraded?
frr	Ribosome recycling	Yes	Yes
ftsZ	Septation initiation	Yes	Not tested
map	Met-aminopeptidase	Yes	Yes
murA	Cell wall synthesis	Yes	Yes
nadD	NAD^+ synthesis	Yes	Not tested
nadE	NAD^+ synthesis	Yes	Yes
rplI	Ribosomal L9	No	Yes
rpoB	RNAP-beta	Yes	Yes
rpsA	Ribosomal S1	Yes	Yes
rpsC	Ribosomal S3	Yes	Yes
selD	Selenide kinase	Yes	Yes
tsf	Elongation factor Ts	Yes	Not tested
yeaZ	Conserved protease	Yes	Yes

[a] *E. coli* strains were generated containing sequences encoding the control and degradation tags on the genes listed in the first column. A brief description of the encoded gene product's function is listed along with its reported essentiality. Degradation was assessed using transduction assays with $clpX^+$ and Δ*clpX* recipients and Western blots that detect the tags upon conditional ClpX or ClpXP expression.

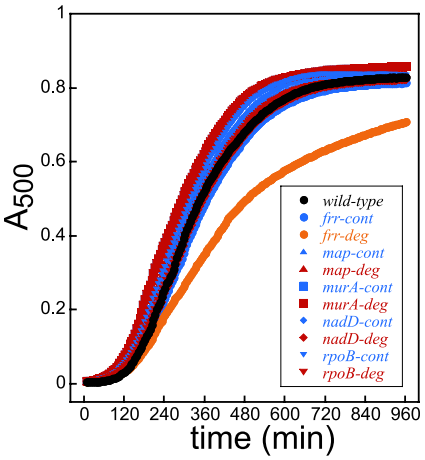

FIG 2 Growth comparisons of strains with degradation or control tags on essential proteins. Turbidity was measured as apparent absorbance at 500 nm in a shaking 96-well plate at 37°C in LB medium. The parental, wild-type (black), target control (blue), and target-deg (red) strains grew similarly. The growth of *frr*-deg (orange) was markedly impaired in comparison to that of the other strains.

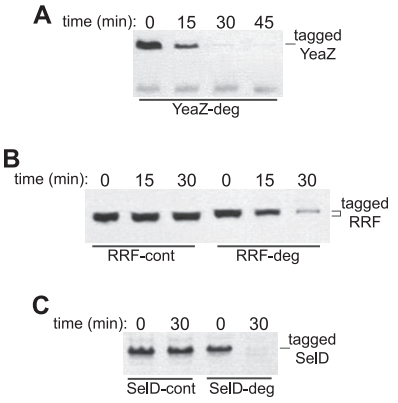

FIG 3 Expression of ClpX leads to the rapid depletion of target proteins. Liquid cultures of strains with tags on YeaZ, RRF, and SelD were induced to express ClpX from a plasmid. Cultures were sampled, and total protein was normalized and analyzed using Western blots directed against the FLAG epitope in the tags. In each case, ClpX expression led to a rapid reduction in the level of protein bearing a degradation tag. (A) YeaZ-deg; (B) RRF-cont and RRF-deg; (C) SelD-cont and SelD-deg.

tently lower efficiency than the control (~25%). In another, *selD*-deg was transduced with the similar frequencies into either *clpX*$^+$ or Δ*clpX* cells, despite it being reported as an essential gene (2).

We transformed the *frr*-deg and *selD*-deg strains with a ClpX expression plasmid so that we could control the timing of exposure to ClpX. For comparison to a target that was previously verified as essential, we also transformed the *yeaZ*-deg strain, because this gene was unable to be transduced into *clpX*$^+$ cells. We grew liquid cultures of each and induced the expression of ClpX in early exponential phase. The presence of the tagged target was monitored in total protein samples at different time points after ClpX expression using Western blots directed against the FLAG epitope in the tag sequence. Consistent with transduction data that indicated YeaZ-deg was a substrate for ClpX, it was rapidly depleted upon ClpX expression and reduced to trace levels within 30 min (Fig. 3A). In the case of ribosome recycling factor (RRF)-deg, the abundance of the target was reduced upon ClpX expression, but approximately 10% remained after 30 min (Fig. 3B). Thus, RRF-deg was a substrate for ClpX, but it was not efficiently depleted. Transductants with reduced activity of essential genes can yield apparently healthy colonies if the residual level of gene product is sufficient or if the restrictive mutation allows slow growth that increases observed reversion or suppression. To distinguish between these possibilities, we sequenced the *frr*-deg, *clpX*, and *clpP* genes in two *clpX*$^+$ *frr*-deg transductants. Each strain still contained the correct degradation tag sequence on the target and contained wild-type *clpX* and *clpP*. If a low level of RRF was sufficient for healthy colony formation, then comparable numbers of *frr*-deg transductants should have been recovered from *clpX*$^+$ and Δ*clpX* recipients. In the following section, we describe the appearance of escape mutants that become refractory to ClpXP-mediated degradation by an unknown mechanism, and this phenomenon is likely the cause of the appearance of "background" colonies when certain essential target-deg genes are transduced into *clpX*$^+$ cells.

A mutant allele of *selD* was previously identified that is defective in selenophosphate production (26); yet, *selD* was listed as essential in a previous report that identified essential genes in *E. coli* by their sensitivity to transposon interruption (2). Our transduction data suggested that SelD was either unable to be degraded by ClpXP, that the residual level of SelD was sufficient to support growth, or that the protein is nonessential. ClpX expression in the *selD*-deg strain resulted in the rapid and thorough depletion of SelD (Fig. 3C). Additionally, there were no observable differences in the growth of strains harboring *selD*-deg and control-tagged *selD* (*selD*-cont) when these strains were plated with *clpX* induced (not shown).

Recent investigations of *selD* suggest that it is dispensable in *E. coli* and that it can be deleted using a targeted approach (W. Self, personal communication). Although our transduction and degradation data are consistent with the idea that SelD is nonessential, residual SelD activity or alternative selenium sources may be sufficient for growth when this target is depleted. A less likely possibility is that the FLAG epitope was cleaved and ClpXP released the remainder of SelD as a functional fragment. Known cases of substrate release by ClpXP typically require reduced ATP levels and an encounter with a very stable domain at the entrance to ClpX (28, 34); yet, the recent crystal structure of *E. coli* SelD indicates that the core of the protein should have been substantially disrupted at the stage where the tag was present in ClpP (35).

Tailoring protease expression and the degradation of ribosomal protein S1. To optimize protease expression and evaluate the ability of our degradation system to deplete a large protein that is highly expressed, we focused on ribosomal protein S1. This 61-kDa, multidomain protein is an essential translation factor that recruits mRNAs during translation initiation (21, 54). In addition to its association with a wide array of mRNAs, S1 also binds to the ribosome and to the degradosome (1, 10, 17, 37, 38). Therefore, for S1 to be efficiently depleted, it must be degraded substantially faster than it is produced and also stripped from a number of macromolecular interactions. S1 levels have been reduced in *E. coli* by two other groups using depletion-by-cell-division strategies wherein the *rpsA* gene was placed under the control of a repressible promoter (9, 47). In those studies, several hours were required to appreciably deplete S1, because, not accounting for

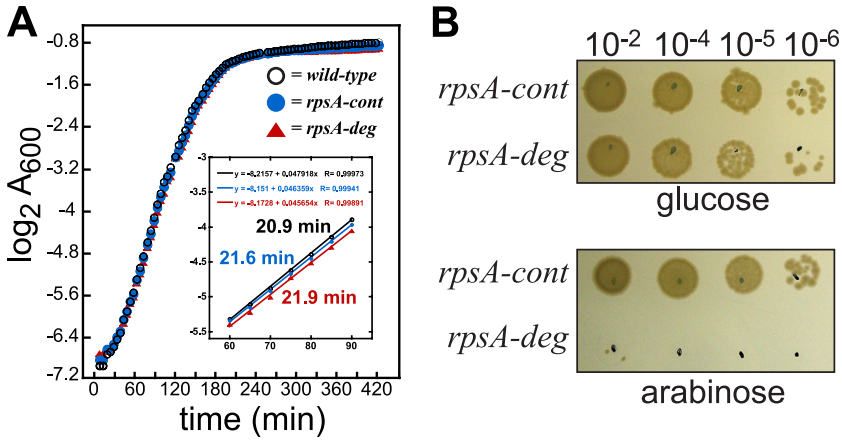

FIG 4 Growth of *rpsA*-tag strains and selection of pClpPX. (A) The growth rates of strains with control and degradation tags on S1 were compared to that of the wild-type parent. Fifty-microliter cultures were grown in shaking 96-well plates at 37°C in LB medium supplemented with 0.2% glucose. The turbidity data were converted to $\log_2$ and plotted as a function of time. The data from the fastest growth stage was fitted to obtain slopes which were used to calculate doubling times (inset). (B) Cultures of *rpsA*-cont and *rpsA*-deg harboring a pClpPX expression plasmid were grown in LB-ampicillin supplemented with 0.2% glucose until early stationary phase and then serially diluted in cold LB. Ten-microliter spots were plated onto LB-ampicillin plates containing either 0.2% glucose or 0.2% arabinose and incubated for 16 h at 37°C. The induction of pClpPX reduced colony formation by ~10^5. The few colonies of *rpsA*-deg that form on arabinose appear refractory to target protein degradation.

natural protein turnover, a single cell division diluted this factor only 2-fold. Moreover, the reduction of this essential factor inhibited cell division, a common problem during conventional depletion experiments.

To determine the influence the C-terminal tags on S1, we measured the fitness of the *rpsA*-cont and *rpsA*-deg strains by comparing their maximum growth rates in rich medium, a condition that places a high demand on the translation system. The wild-type and *rpsA*-cont strains grew with nearly identical maximal rates, and the *rpsA*-deg strain exhibited a slight growth rate reduction (Fig. 4A). Therefore, the addition of these tags to S1 did not substantially impair its essential functions in translation.

In preliminary experiments, we had difficulty matching ClpX expression to certain targets, such as S1. In short, expressing protease from a low-copy-number plasmid did not provide rapid depletion, and using high-copy-number plasmids caused leaky expression that substantially reduced the growth of target-deg strains prior to induction. To resolve these issues, we developed a plasmid library containing both the *clpP* and *clpX* genes under the control of the *araBAD* promoter. The library contains two randomized nucleotide positions in the ribosome-binding sites for each gene such that the expression levels and ratio of ClpP and ClpX were variable for each clone (45). The *rpsA*-deg strain was transformed with this library, and individual colonies were then test streaked on plates containing glucose or arabinose to either repress or induce ClpXP expression. Clones were selected that exhibited healthy growth on glucose but that were restricted on arabinose. The pClpPX plasmids were isolated from these strains and used to transform the *rpsA*-cont strain for comparative studies (Fig. 4B).

We observed that there was a background of *rpsA*-deg cells harboring ClpXP plasmids that formed large colonies when plated on arabinose, suggesting that S1-deg became refractory to degradation. Because we intended to monitor the responses to target depletion in large cultures, having a high percentage of cells not undergoing S1 degradation could present a complication. Therefore, using a ClpXP plasmid that severely restricted the growth of *rpsA*-deg when induced, we determined the escape frequency by plating dilutions of stationary-phase cultures on glucose and arabinose. We reproducibly observed colony formation by *rpsA*-deg at ~1×10^{-5} in comparison to the same culture on glucose plates and to the *rpsA*-cont strain (e.g., Fig. 4B). Considering that the *rpsA*-deg strain harboring the plasmid grew well prior to induction and that the protease was encoded on a multicopy plasmid, the observed escape frequency appeared too high to be accounted for by spontaneous mutation of *clpP* or *clpX*. Nonetheless, we resequenced *clpX*, *clpP*, and the *rpsA*-deg genes from two independent large colonies, and no mutations were detected. Therefore, this degradation system is sufficient to restrict growth in a large majority of the culture, which makes it suitable for biochemical analyses, but escape from target degradation can occur at high frequency that restricts certain genetic studies. For these reasons, we now take precautions when handling target-deg strains to prevent premature protease induction, such as growing in the presence of excess glucose and not allowing cultures to stand in stationary phase.

Degrading S1 leads to a rapid reduction in growth rate. If a target protein is responsible for the production of an essential factor, a prediction can be made that cell division should continue for some time after the depletion of the target, because the factor would persist, and cell division would be needed to deplete the product as well. On the other hand, if the target protein is directly linked to a cell growth process, then target depletion is expected to have an immediate effect on growth rate. To determine the effect of S1 depletion on cell growth, we monitored the growth of the *rpsA*-deg strain harboring a ClpXP plasmid that greatly restricted colony formation when plated on arabinose. Several cultures were inoculated that contained 0.2% arabinose and various concentrations of glucose. Upon consumption of the preferred glucose, the cultures responded to the presence of arabinose and autoinduced the expression of ClpP and ClpX (48). In each case, protease expression caused a rapid reduction in growth rate, with the effect being more pronounced at lower glucose concentrations (Fig. 5A). In a separate experiment, the protease system was man-

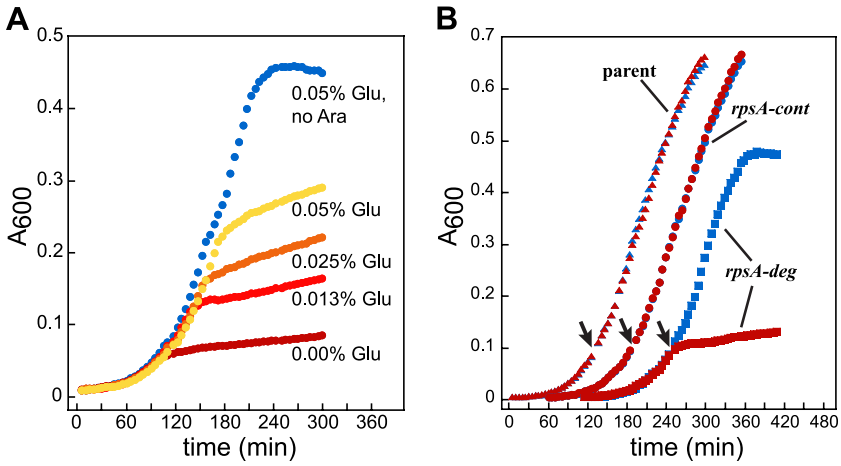

FIG 5 Induction of pClpPX causes a rapid reduction in the growth rate of *rpsA*-deg cultures. (A) An overnight culture of *rpsA*-deg grown in glucose was diluted 100-fold into LB-ampicillin medium containing differing concentrations of glucose and 0.2% arabinose. Upon depletion of the preferred glucose, the arabinose promoted ClpXP expression at different culture densities (autoinduction). In the absence of glucose, early growth was comparable to cultures with glucose before a transition to a slow, linear growth rate, which suggests that the arabinose present did not fully induce protease expression during this phase. In the absence of arabinose, this strain does not reach high culture densities unless there is saturating glucose present. (B) Cultures of the wild-type parent and *rpsA*-cont and *rpsA*-deg strains, each harboring a pClpPX plasmid, were grown in LB-ampicillin with 0.2% glycerol. In exponential phase, the plasmids were manually induced by the addition of 0.2% arabinose (arrows). The growth curves are offset by 60 min for clarity. Expression of the protease did not restrict the growth of the parent or *rpsA*-cont strains but did cause a rapid reduction of growth of the *rpsA*-deg culture.

ually induced in *rpsA*-cont and *rpsA*-deg cells by the addition of arabinose while they were in exponential phase. The growth reduction was pronounced, with a sharp transition into a slow, linear rate within 5 min (Fig. 5B). In this experiment, a lack of glucose in the uninduced *rpsA*-deg culture led to a cessation of growth at a lower cell density than controls, presumably because the protease plasmid became derepressed in depleted medium. Below, we show that total S1 levels do not appreciably decline until 15 min after protease induction, which suggests that growth rate may be linked to S1 longevity and not necessarily to overall S1 abundance.

ClpXP expression removed the existing pool of S1 and maintained S1 depletion. We manually induced the expression of ClpXP in wild-type, S1-cont, and S1-deg liquid cultures and sampled them at 10-min time points (a logistic limit) to measure cell density, to isolate total protein, and to isolate total RNA. The turbidity measurements revealed growth rate responses that were comparable to those shown in Fig. 5B: protease expression in the *rpsA*-deg strain led to a sharp transition into slow, linear growth (not shown). Protein samples were normalized to each other, and the abundances of S1-cont and S1-deg were monitored by Western blotting with anti-FLAG antibody. Induction of the ClpXP plasmid in the *rpsA*-cont strain did not affect the levels of S1 (Fig. 6). In contrast, the level of S1-deg dropped to approximately 50% by 15 min and was reduced to trace levels by 25 min. A separate quantitative Western analysis containing serial dilutions of the preinduced sample revealed that the level of S1 in the 25-, 35-, and 45-min samples was less than 0.5% of the normal amount (not shown). Additionally, when run on the same Western blot, the amounts of S1-cont and S1-deg in the preinduction samples were the same (not shown). Therefore, induction of the degradation system led to a rapid and thorough depletion of S1-deg.

S1 depletion induces rapid and differential changes in mRNA levels. The brisk removal of S1 in our system provided a unique opportunity to detect any immediate changes in the abundance of mRNAs, with the intent of identifying direct influences of S1. We placed focus on several mRNAs: that which encodes S1 itself (*rpsA*), those encoding proteins that physically interact with S1 (S2 [*rpsB*], RNase E [*rne*], PNPase [*pnp*]) (1, 10, 17, 37, 38), messages known to be degraded by the degradosome and stabilized by S1 (CspE and S15 [*rpsO*]) (9, 16, 40), a collection of unrelated mRNAs with diverse 5′ untranslated regions (UTRs) and translation initiation features (encoding LacI, LacZ, OmpT, and RecA), and two housekeeping transcripts (encoding GAPDH [*gapA*] [11, 43, 49, 51] and CsdA [36, 44]).

Total RNA was purified from each of 12 experimental samples described above and converted to cDNA. The abundance of each mRNA was measured in each using qualitative reverse transcription (qRT)-PCR with primers that amplified the 5′ ends of each open reading frame. Relative comparisons were made to the amounts present prior to the induction of ClpXP and to a separate culture also induced to express ClpXP but containing wild-type S1. Because each culture was sampled six times for independent cDNA libraries, trends in transcript levels became readily appar-

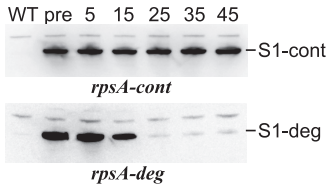

FIG 6 Expression of ClpXP causes a rapid depletion of S1-deg. Cultures of *rpsA*-cont and *rpsA*-deg strains were grown to exponential phase in LB-ampicillin and 0.2% glycerol and then induced to express ClpXP by the addition of 0.2% arabinose. Five minutes prior to induction and at 10-min intervals, samples were withdrawn, and total protein was prepared for Western analysis using anti-FLAG antibody. Protein from a wild-type culture was also analyzed to reveal nonspecific bands. Parallel samples were also withdrawn for RNA preparations which were used for the qRT-PCR analyses.

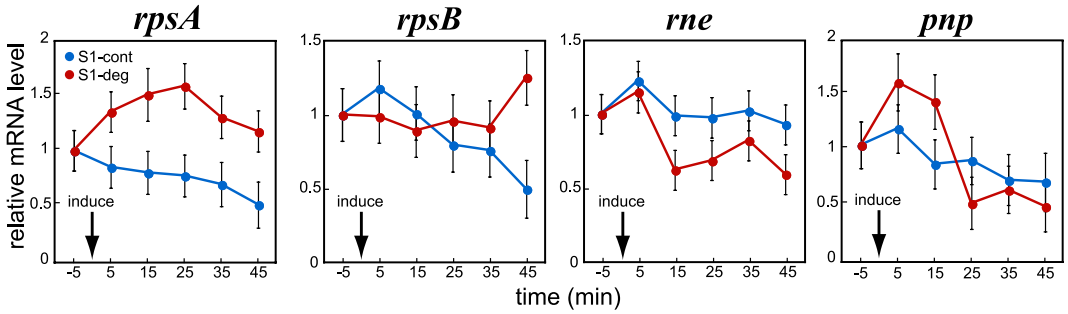

FIG 7 Transcripts encoding S1 and proteins that interact with it. The levels of *rpsA*, *rpsB*, *rne*, and *pnp* mRNAs upon ClpXP induction were measured using qRT-PCR and plotted relative to the amounts present prior to protease expression. In the culture undergoing S1 depletion, the *rpsA* transcript was elevated relative to the control. Aside from the last measurement of the *rpsB* message, the other transcripts were not substantially affected by S1 depletion on this time scale.

ent. Moreover, each library was used for the measurement of all tested transcripts, so consistent, differential changes between unrelated transcripts in the same samples served as convenient internal controls.

In general, in the S1-cont cultures, the induction of ClpXP had little to no effect on transcript levels. The abundance of some tested transcripts in the S1-cont culture declined modestly as the cells exited exponential phase, which may reflect their being downregulated as the medium depleted. Likewise, the abundance of the *rpsA* transcript encoding S1 declined in the control culture. In contrast, the level of this transcript in the culture undergoing S1 degradation increased and remained elevated in comparison throughout the experiment (Fig. 7). Therefore, consistent with previous reports, the *rpsA* transcript level was inversely influenced by the levels of S1 (8, 39, 46).

The messages encoding factors that physically interact with S1 exhibited differential changes in abundance upon S1 depletion. The *rpsB* transcript, encoding a protein thought to be a component of the S1 docking site on the ribosome (S2) (30), remained at a level comparable to that in the preinduced culture. The levels of the messages encoding two degradosome components (RNase E and PNPase) were present at levels that were similar to those in the control culture, with perhaps a slight reduction at time points where S1 was substantially depleted (Fig. 7). Therefore, there was no apparent correlation between the abundance of transcripts encoding proteins that interact with S1 and the level of S1 itself.

The *ompT*, *recA*, *lacZ*, and *rpsO* transcripts each exhibited marked changes in abundance in the culture undergoing S1 depletion relative to the control, but the trends were not consistent: the *ompT*, *recA*, and *lacZ* messages declined, while that of *rpsO* remained essentially constant. At the same time, the *lacI* and *cspE* mRNA levels remained indistinguishable from those in the control culture (Fig. 8). Thus, some mRNAs are influenced by the presence of S1, while others are apparently unaffected.

We chose to also analyze the *gapA* transcript, because GAPDH expression is considered to be constitutive (11, 43, 49, 51), and the message encoding the CsdA cold-shock factor, because it is a multicopy suppressor of a defective S2 protein that indirectly influences S1's ability to associate with ribosomes (23, 30, 50). Of the transcripts analyzed, the most striking examples of differential changes in mRNA abundance were for these two messages. During the time that S1 levels rapidly declined (from 5 to 15 min), the level of message encoding GAPDH dropped more than 20-fold, while the amount of message encoding CsdA increased 5-fold (Fig. 8). Therefore, some messages appear hypersensitive to the presence of S1, exhibiting rapid changes in abundance that are greater than those typically encountered during depletion studies. Considering that the abundances of these two mRNAs changed in quick step with the loss of S1 and that they play important roles in maintaining cell physiology, it is tempting to speculate that they have design features that make them particularly sensitive to the amount of available S1 or to translation initiation efficiency.

DISCUSSION

We demonstrate a targeted degradation system that allows for the rapid depletion of proteins upon the tailored expression of a processive protease. This system differs from other targeted degradation systems primarily in that a rapid depletion of a broad range of proteins can be achieved without complications that arise using delivery adapters that affect the K_m of the target for the protease or promote multimerization. By rapidly depleting a substantial portion of the existing protein pool, a unique cell biochemistry is created that leads to responses related to the function of the targeted protein, a process we term "cell reflex." The reflex is expected to include changes to those factors that are in rapid flux, such as mRNAs and other metabolites affected by the perturbed biochemical pathway.

As with other targeted degradation strategies, the activity of ClpXP currently restricts the use of the system to cytosolic proteins, and we have not evaluated inner-membrane targets with established cytosolic C termini. Another point that must be stressed is that ClpXP has a collection of other proteins that it can engage (3, 19), so physiological responses must be compared to control cultures undergoing ClpXP expression without target depletion, and synergistic effects must always be considered when characterizing a response.

Our focus on S1 was inspired by previous studies in which this essential factor had been depleted using the traditional approach of subcloning the gene under a controllable promoter and then turning off expression of the gene. Those studies confirmed that S1 was essential and revealed that prolonged depletion led to a global loss of protein synthesis with a bias toward the translation of ribosomal proteins (47). Apart from a direct role in recruiting mRNAs, slow S1 depletion by cell division caused a drop in the levels of many mRNAs, presumably because they became more susceptible to RNases in the absence of engagement with the translation machinery (9). Our rapid depletion of S1 revealed that mRNA levels are not uniformly affected: some increased, some

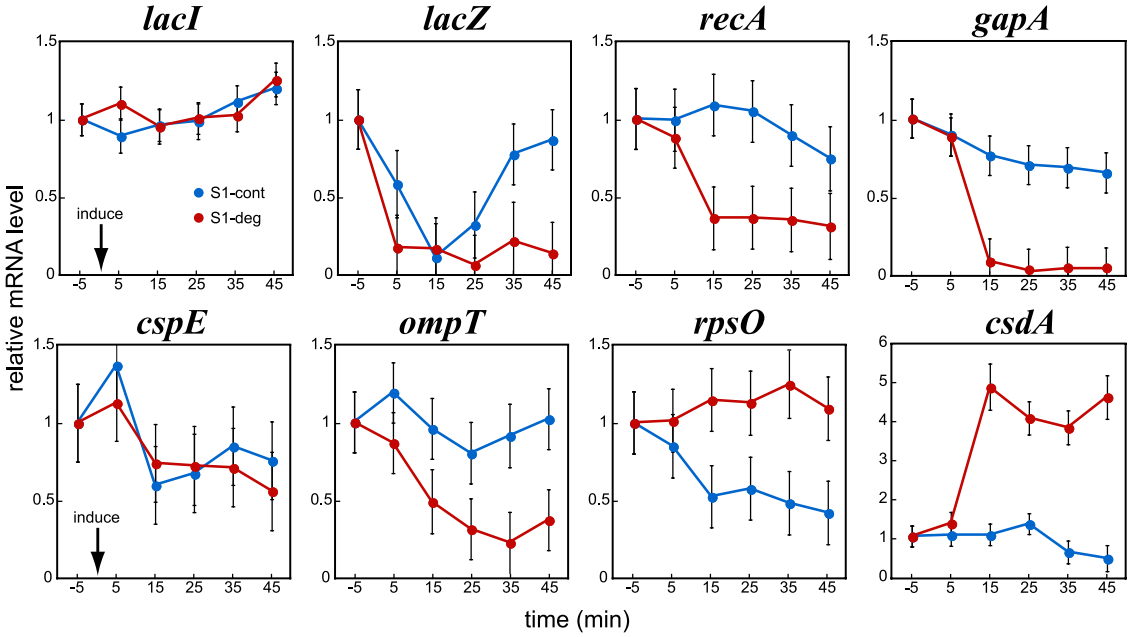

FIG 8 Transcripts encoding proteins unrelated to S1 function. The levels of *lacI*, *lacZ*, *recA*, *gapA*, *cspE*, *ompT*, *rpsO*, and *csdA* mRNAs upon ClpXP induction were measured using qRT-PCR. Some transcript levels were affected by the depletion of S1, while others were not. The *lacI* message exhibited the least variance of all transcripts tested. The decline and recovery of the level of the *lacZ* transcript in the S1-cont culture was reproducibly observed and may reflect an effect of ClpXP induction (the decline in both S1-cont and S1-deg began before appreciable S1 depletion). The *gapA* and *csdA* transcript levels changed the most, with a >20-fold reduction and ~5-fold increase, respectively.

decreased, and some stayed the same. Thus, the presence of S1 directly influences some but not all transcripts. Clearly, more detailed biochemical studies are needed to reveal the molecular details of these differential changes, because the qRT-PCR measurements reported only on the presence of the 5′ end of each ORF. However, it is likely that changes in transcription, mRNA stability, or both are responsible. It is notable that, relative to other mRNAs, the ribosomal protein transcripts we monitored appeared more stable in the absence of S1, and this phenomenon may be responsible for the translation biases observed upon S1 depletion in prior studies (47).

Unraveling direct from indirect effects during protein depletion experiments can be challenging, especially when factors are depleted that may influence the activity of toxins or stress responses. For other studies, we aim to deplete essential proteins as fast as possible in the hopes we can tease out direct from indirect influences of targets. During the evaluation of this system, we intended to use the *gapA* transcript as a control in our cDNA libraries because it is commonly used as a constitutive marker (11, 43, 49, 51). Surprisingly, however, the level of *gapA* mRNA dropped precipitously as S1 was depleted. Because this drop occurred before cell division could have appreciably reduced GAPDH levels, and because the *gapA* transcript is more stable than the average mRNA when transcription is blocked (5, 49), we suggest that the turnover of this mRNA may be a link between translation initiation and carbon metabolism. Likewise, the striking increase in the mRNA encoding the CsdA cold-shock factor may reflect an evolved signal that reports on the efficiency of engagement of mRNAs by the ribosome.

We developed this targeted degradation system with an eye toward discovering concomitant physiological changes. By monitoring a larger data set, perhaps with global transcriptome analysis or metabolite profiling, it is feasible that many pathways affected by the depletion of a particular target can be identified during a single experiment. Such data would not only reveal coregulated systems but would also provide strong clues about gene functions that are yet undiscovered.

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