ClpX, an Alternative Subunit for the ATP-dependent Clp Protease of *Escherichia coli*

**SEQUENCE AND IN VIVO ACTIVITIES**

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The ATP-dependent Clp protease of *Escherichia coli* consists of two subunits, the ClpP subunit, which has the proteolytic active site, and ClpA, which possesses ATPase activity and activates the proteolytic activity of ClpP in *vivo*. Recently, Zylczik and co-workers (Wojtkowiak, D., Georgopoulos, C., and Zylczik, M. (1993) J. Biol. Chem. 268, 22609-22617) identified another *E. coli* protein that activated ATP-dependent degradation of a *λ* protein in the presence of ClpP. The amino-terminal sequence of this protein corresponds to the translated amino-terminal sequence of a gene that we have named clpX. ClpX encodes a protein with M, 46,300, similar to that observed for the protein purified by Wojtkowiak et al. *clpX* is in an operon with *clpP*; both genes are cotranscribed in a single heat-inducible 2200-base mRNA, with *clpP* the promoter proximal gene. The sequence of ClpX includes a single consensus ATP-binding site motif and has limited homology to regions of ClpA and other members of the ClpA/B/C family. A third group of proteins, ClpY, closely related to ClpX, has been identified by sequence homology. Mutations in either *clpX* or *clpP* abolish degradation of the highly unstable *λ* protein *in vivo*. *clpX* mutants are not defective in degradation of previously identified ClpA/ClpP substrates such as a ClpA-β-galactosidase fusion protein. It appears that selectivity of degradation by ClpP *in vivo* is determined by interaction of ClpP with different regulatory ATPase subunits.

Energy-dependent proteolysis degradation in both prokaryotes and eukaryotes accounts for the major portion of degradation of abnormal proteins and specific, unstable proteins. In *Escherichia coli*, two ATP-dependent proteases have been characterized: the Lon protease (or La), and Clp protease (or Ti) (see Ref. 1, for recent review). Clp consists of two subunits, one of which is capable of degrading peptides in an energy-independent manner (ClpP) (2) and the other of which possesses ATPase activity (ClpA) (3-5). In the presence of ATP, these subunits assemble to form a protease complex which degrades cassein in an ATP-dependent manner (6). Genes encoding proteins homologous to ClpP have been identified in the chloroplast genomes for many plants, and genes encoding proteins with extended similarity to ClpP have been identified in prokaryotes and eukaryotes (7, 8). *E. coli* has at least one such homolog of ClpA, called ClpB. ClpB has a protein-stimulated ATPase activity, but there is as yet no evidence that ClpB can combine with ClpP to carry out ATP-dependent proteolysis (9).

The genes encoding ClpP, ClpA, and ClpB are widely separated from each other on the *E. coli* chromosome. Both clpB (mapping at 58 min) and clpP (at 10 min) are at least in part regulated by a sigma-32 dependent heat shock promoter, although clpA (at 20 min) does not seem to be (4, 10-12). Evidence that ClpA and ClpP work together *in vitro* as well as *in vivo*, is provided by the observation that a clpA-lacZ protein fusion is degraded in Clp* cells* but is stabilized in cells devoid of either ClpA or ClpP (4, 13). Intracellular turnover of two other fusions is also dependent on both ClpA and ClpP. Varshavsky and co-workers (14, 15) have reported that β-galactosidase constructs containing abnormal amino-terminal amino acids can be rapidly degraded in *E. coli* in a process which is dependent on both ClpA and ClpP.

However, not all activities dependent on ClpP *in vivo* are also dependent on ClpA. The NH₄-terminal 14 amino acids of ClpP are cleaved off during assembly of the protease; this activity is dependent on a functional ClpP, but is apparently normal in clpA mutants (8). Some growth defects have been observed in cells carrying ClpP mutations in certain backgrounds; clpA mutations do not show the same defects and in fact may help relieve the clpP defects. Toussaint and co-workers (16) have recently shown that Muvir repressor is degraded in *E. coli* and stimulates the rapid degradation of an endogenous wild-type repressor after infection of Mu* lysozyme; this degradation is dependent on ClpP but not ClpA. Therefore, additional proteins capable of working with ClpP may exist in *E. coli*. The work presented by Zylczik and co-workers (17) in this volume provides the most direct evidence for this thus far. They identified a number of proteins able to carry out ATP-dependent degradation of *λ* protein; detection of one of these protease activities requires ClpP and a unique subunit, originally called LopC (17). As part of a collaborative effort to identify the gene encoding LopC, we determined that the NH₄-terminal sequence of the LopC protein was identical to that for the predicted protein sequence of the gene just downstream of clpP, which we have called *clpX*. We show here that ClpX plays a central role in the *in vivo* degradation of *λ* protein. ClpX appears to represent a new class of Clp ATPase subunits.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, Bacteriophage, and Plasmids*

Isogenic strains containing single *clp* mutations or a lon mutation were constructed by PI transduction, selecting the antibiotic resistance.

1. M. Maurizi, unpublished observations.
2. S. Gottesman and L. Wellen, unpublished observations.
3. V. de Crecy, unpublished observations.
ance marker within the gene. clpP::cat (13), clp319::an (4), clpX::kan (12), and lon-164::Del18 (18) have been previously described. The isolation of clpX::kan is described in this paper. The starting strain for the canavanine protein turnover experiments was SG1101, a wild-type E. coli strain carrying a *arg* mutation (4). Recipient strains for the O protein turnover experiments were all derivatives of SG20250, a relative of MC4100 (19) lysogenized with λc857Sam7 or λc857. SG12054 is a clpX::cat derivative of M21 (20).

Cloning and Sequencing of clpX

pWPC9, carrying *clpP* and the region downstream of *clpP* up to the beginning of *lon*, has been described (13). The EcoRV-HindIII fragment from pWPC9 was cloned into both M13mp18 and M13mp19 vectors for dyeoxy DNA sequencing (22). Both the universal M13 primers and appropriate primers from within the sequenced region were used. The sequence for the whole gene was determined on both strands, using reactions with dITP in regions that showed compression. The sequence shown in Fig. 2 starts 1 base pair beyond the translation termination codon of *clpP* and extends to the region just ahead of the *lon* promoter. A portion of the 5' end of clpX has been published previously (13), as has the 3' portion of the gene (23). A recent map of the E. coli chromosome (24) shows the location of clpX, based on our work; all assignments of *E. coli* genes to particular minutes are based on the centosome assignments used in that map.

**Mutagenesis of clpX**

pWPC9, containing both *clpP* and clpX, was digested with Ncol and religated. This generated a plasmid, pWPC51, that lacked 745 base pairs of the clpx gene (deleting by about 65% of sequence shown in Fig. 2 to bp 1250). A 12-nucleotide double-stranded oligomer (CATGGCTGCAGC) containing a PstI site and overhanging NcoI sticky ends was ligated with Ncol cut pWPC51 to create pWPC52. A *BamHII*HindIII fragment containing the interrupted clpx gene as well as the rest of the original bacterial insert from pWPC9 was ligated with *BamHII*HindIII digested pACYC184, to create pWPC53. This plasmid was digested with PstI and ligated with a cassette encoding kanamycin resistance, made by digesting pUC4K with PstI (25). The *ΔclpX*-kan plasmid, pWPC54, was linearized with *Poul* and *BamHII* and the digested DNA transformed into JC7623, a recBC strain obtained from N. Truneh. The recombinant plasmid was not detected by in vivo linear DNA and is able to recombine it into the chromosome. Colonies which were kanamycin resistant but ampicillin sensitive were purified and selected for further testing. One such isolate was called SG1152. Pl1 grown on this strain was used to introduce the clpX mutation into a variety of other backgrounds.

**Purification of ClpX for Production of Antibodies**

ClpX was produced from a plasmid with the clpX genes under control of the p promoter, plasmid lambda in SG12054, a host cell carrying the heat-sensitive lambda repressor, clpX57. The plasmid pSK20 used was a derivative of the expression plasmid pRE1 (26) created by S. K. Singh in the following way: an *Ndel*-BamHI fragment from pWPC22, which carries *clpP* lacking the coding region for the first 13 amino acids, was inserted in the multiple cloning site of pRE1. Then, a double-stranded oligonucleotide coding for the first 13 amino acids but altered to include an *Ndel* site at the initiator methionine codon was inserted at the *Ndel* site orientation was confirmed by DNA sequencing. An oligonucleotide linker carrying an SstI site was inserted at the *BamHI* site. Finally, an *MluI*-SstI fragment from pWPC9 carrying the second half of *clpP* and the entire *clpX* gene was inserted between the *MluI* site in clpX and the SstI site.

For expression of ClpX, cells were grown to a density of 1.5 OD060 units and induced for 2 h at 42 °C. ClpX produced under these conditions was purified to homogeneity by inclusion body extraction. The inclusion bodies were washed with buffer and extracted with 1% sodium dodecyl sulfate (SDS). The extract was run on a 12% acrylamide gel in SDS, and the ClpX band was electroeluted. Antibodies were raised in a rabbit after two injections of 0.5 mg each in Freund's adjuvant as described previously (4).

**Western Blot Conditions**

To detect the accumulation of Xo protein in various mutants, cells carrying the λc857Sam7 prophage were grown in tryptone broth to 0.5 OD060 units at 32 °C to a density of about OD060 = 0.5 and transferred to 42 °C. Samples were taken after 0, 30, and 60 min and quenched with 5% trichloroacetic acid. Preparation of cellular protein for SDS gel analysis and Western blotting procedures were described previously (4). O protein standard was the gift of R. McMacken and rabbit anti-O protein antibody was a gift of S. Wickner. ClpX was detected in cells precipitated in trichloroacetic acid, run on SDS gels, and blotted to nitrocellulose.

**In Vivo Tests of Protein Degradation**

ClpX is encoded by a prophage, which carries the *supF* amber suppressing tRNA under the control of the *clp* promoter, were grown in TBMM (tryptone broth with 0.0001% vitamin B1, 0.01 M MgSO4, and 0.2% maltose) in the presence of 0.02% isopropyl-1-thio-β-D-galactopyranoside overnight to induce the suppressing tRNA. Cells were collected by centrifugation and resuspended in 0.1 M MgSO4, 0.1 ml of cells was adsorbed for 10 min at room temperature with 10 μl of imm434 Oam29 or imm434 Nam7Nam53 phage to give a multiplicity of infection of less than 0.1, and then diluted 100-fold into LB (27) with 0.01 M MgSO4. The samples were incubated at 42 °C for 15 min, chlorofrom was added, and the phage titer determined by plating on the permissive host LE392 (supF E. coli) (28).

**Burst Size Experiments**—Cells lysogenic for the imm21plac-supF phage, which carries the supF amber suppressing tRNA under the control of the *clp* promoter, were grown in TBMM (tryptone broth with 0.0001% vitamin B1, 0.01 M MgSO4, and 0.2% maltose) in the presence of 0.02% isopropyl-1-thio-β-D-galactopyranoside overnight to induce the suppressing tRNA. Cells were collected by centrifugation and resuspended in 0.1 M MgSO4, 0.1 ml of cells was adsorbed for 10 min at room temperature with 10 μl of imm434 Oam29 or imm434 Nam7Nam53 phage to give a multiplicity of infection of less than 0.1, and then diluted 100-fold into LB (27) with 0.01 M MgSO4. The samples were incubated at 42 °C for 15 min, chlorofrom was added, and the phage titer determined by plating on the permissive host LE392 (supF E. coli) (28).

**Northern Blot Methods**

Total RNA was isolated from *E. coli* cells grown at 32 and 42 °C to a density of 0.5–0.8 OD060 units using the procedure described previously (13). A 1.1-kb agarose gel was run under denaturing conditions with about 5 μg of each RNA in a lane and blotted to nitrocellulose as described (29). The blot was prehybridized for 4 h at 42 °C with Hybridol 1 (Oncor) and then hybridized with about 50 × 106 disintegrations/min of heat-denatured labeled primer made by random hexamer priming. The *clpF* - or *clpX*-specific DNA templates for the random primer were made by the polymerase chain reaction protocol (30) and corresponded to bases 260–950 in the *clpF* sequence and bases 181–1180 in the *clpX* sequence (Fig. 1). After hybridization, the blots were washed twice in 2 × SSC, 0.1% SDS at 42 °C and then twice at 62 °C in 0.1 × SSC, 0.1% SDS for 15 min each. The blots
RESULTS

Sequence of clpX—clpP maps at 10 min on the E. coli map, slightly upstream of lon, the gene for the other major ATP-dependent protease of E. coli. Between the two genes is a region of about 1500 base pairs, which appears to be part of the clpP operon and encodes a protein of 424 amino acids (Figs. 1 and 2). We have called this gene clpX. The DNA sequence for the entire clpX gene and the translated amino acid sequence for the ClpX protein are given in Fig. 2. The ClpX open reading frame begins 125 bases downstream from the translation codon for ClpP. There is no obvious promoter sequence in the 5' upstream region and no obvious transcription terminator between the clpP and clpX genes. There is an inverted repeat downstream of the open reading frame for ClpX, suggestive of a ρ-independent transcription terminator (underlined in Fig. 2). ClpX has a predicted Mr of 46,300.

Given the possibility that a gene in the same operon with clpP might be related to the function of ClpP, we compared the NH2-terminal sequence of the M, 46,000 LopC protein, found by Zylcz and co-workers to degrade λO protein in the presence of ClpP (accompanying paper by Wojtkowiak et al. (17)), with the sequence for the amino-terminal portion of the ClpX protein predicted from the DNA sequence we obtained for clpX (Fig. 2). There is precise agreement between the NH2-terminal sequence found for LopC and the first 13 amino acids predicted for ClpX, if the NH2-terminal Met after synthesis. Therefore, ClpX is the same as hpC; both are part of a larger ATP-dependent protease complex; this subunit shows a consensus ATP-binding site sequence (boxed in the ClpX sequence in Fig. 2) which is very similar to those found in the ClpA/B/C family of proteins. While the ClpX site can be aligned with either the front or back half of ClpA, it shows a somewhat more extended alignment with the second ATP consensus site of ClpA (Fig. 3B).

In addition, their COOH termini, the sequences of ClpX, Y, A, and B have two extended regions of similarity which include two short sequences (boxed Tail Motifs A and B in Fig. 2) that are identical in all members of the ClpA/B/C family (7, 32).

When the ClpX protein sequence was compared to the sequences of other proteins in the database using the Blast program (33), the best matches were to two related proteins identified as chaperone-like proteins for secretion, cdc48 and a valosin-containing protein (94); this alignment is primarily around the ATP consensus binding site (data not shown).

Recently, Rechsteiner and co-workers (35) reported the sequence of Subunit 4 of the 26S eukaryotic ATP-dependent protease complex; this subunit shows a consensus ATP-binding site and homology to the cdc48/valosin family as well as limited homology to ClpA. Therefore, it appears that ClpA and its relatives ClpX and ClpY are part of a larger ATPase superfamily which includes proteins involved in energy-dependent degradation in eukaryotic cells.

Close to the amino terminus of ClpX is a region unique to the two ClpX proteins with the predicted structure of a single Zn finger. This sequence motif, CxxC (x18) CxxC (boxed and marked as Zn Motifs A and B in Fig. 2), is similar to those found in the DNA-binding domain of hormone-responsive receptor proteins (see Ref. 96, for review of Zn finger domains in hormone-responsive receptors). The function of these clusters of cysteine residues in ClpX is not known.

Mutation of clpX—A deletion-substitution mutation in clpX was constructed as described under "Experimental Procedures," and crossed from the plasmid into the chromosome. PI was used to transduce the clpX::kan mutation from SG1152, the linear transformation host, into hosts carrying clpP::cat mutations or proC mutations. proC is about 20% linked to the clpP-lon region. Among kanamycin-resistant transductants into the clpP::cat host, more than 95% had lost the chloramphenicol resistance marker. When Pro + recombinants of a proC host were selected, 8% were kanamycin resistant; a parallel experiment with a clpP::cat donor gave 16% chloramphenicol-resistant transductants among Pro + recombinants (data not shown). Therefore, the kanamycin resistance marker shows the expected location very close to clpP, and

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Fig. 1. Map of the clpX region of E. coli. A. known contiguous genes in the region of clpP and clpX in the E. coli chromosome. This region corresponds to centisomes 9.9-10.0 of Ruddy (24), clpX is at kilobases 464-466 on Ruddy’s version of the E. coli restriction map. The map was constructed from known sequence information and information presented in this paper. B. expanded view of the region containing clpP and clpX showing restriction enzyme sites used in this paper. F, EcoRV; E, EcoRI; N, NolI; H, HindIII, the extent of DNA deleted in the clpP::cat and clpX::kan deletion/insertion mutations (open bars), and the region covered by the probes used for Northern blotting (checkered bars). The heavy black arrows show the extent of DNA transcribed for the clpXP and lon messages, and the striped arrows show the positions and sizes of the open reading frames. Approximately to scale.

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* Highlander, S., and Weinstock, G. (1993) Infect. Immun. 61, in press.

** F. Slack and A. L. Sonenshein, unpublished observations.
FIG. 2. Sequence of clpX. DNA sequence beginning just beyond the termination codon of clpP and extending to the region upstream of OR. The single letter amino acid code was used for the clpX open reading frame. Boxed amino acid sequences show motifs discussed in the text or Fig. 3; letters in bold face type are highly conserved sequences within these motifs. The underlined nucleotide sequences form an inverted repeat (possible transcription termination sequence). The boxed nucleotide sequences indicate NcoI sites used to generate the deletion/insertion clpX::kan mutation.

linkage to proC is in the range expected, suggesting that clpX mutations are not detrimental to growth of these strains of E. coli under these conditions.

Analysis of Transcription and ClpX Protein Synthesis—Northern blot analysis of RNA from wild-type cells revealed a single major transcript of about 2400 bases that hybridized to both clpP-specific and clpX-specific probes (Fig. 4). A transcript >2 kb encoding ClpP was also observed in exponentially growing cells by others. The amount of the transcript in cells grown at 42 °C was about 2-fold higher than

1 L. Simon, personal communication.
FIG. 3. **Conserved regions within ClpX.** A, schematic representation of ClpX, ClpY, and ClpA. The black bars represent ATPase consensus sequence part A (53); the dark gray bars represent ATPase consensus sequence part B. The striped region shows an extra domain in ClpY, not present in ClpX. **Shaded bars** in the tail region show the positions of highly conserved sequences noted in part B, alignments for conserved motifs in ClpX. The single letter amino acid code was used. The ClpX sequence from *E. coli* (determined here), and from *A. vinelandii* (referred to as open reading frame 9 by Jacobson et al. (51)), the sequences for the COOH-terminal halves of *E. coli* ClpA starting at amino acid 410 (21) and *E. coli* ClpB starting at amino acid 500 (7) were aligned using the Pileup program of GCG (54). A portion of that alignment is shown here; the amino acid numbers above the alignment are those for ClpX. Those positions conserved (score >0.5, from the Dayhoff PAM-250 matrix) in all four sequences are shown in **capital letters** and either the majority amino acid or, where no majority exists, that in ClpX is indicated on the consensus line. The sequences of the ATP-binding site consensus are shown in **bold letters**, as are the highly conserved triplet sequences found in the tail region.

**FIG. 4. Transcription in clpP and clpX mutant hosts.** Northern blot analysis of transcription in the clpP-clpX region. RNA from cells grown at 32 or 42 °C was separated on a 1.1% agarose gel, transferred to nitrocellulose, and probed with 32P-labeled DNA specific for *clpP* (lanes a-f) or *clpX* (lanes g-i) as indicated in the figure. Wild-type cells at 32 (lanes a and g) and 42 °C (lanes b and h); clpP:cat cells at 32 (lanes c and i) and 42 °C (lanes d and f); clpX-kan cells at 32 (lanes e and k) and 42 °C (lanes f and l).
ClpX Subunit of Clp Protease

Table I

| Relevant genotype | Canavanine protein degradation* |
|-------------------|---------------------------------|
| Wild-type         | 100                             |
| clpA              | 85 ± 7                          |
| clpP              | 83 ± 3                          |
| lon               | 66 ± 7                          |
| lon clpP          | 25 ± 1                          |
| lon clpX          | 56 ± 7                          |

* Isogenic derivatives of Δarg E. coli K12, strain SG1101.

Degradation rate in the wild-type strain was 32%/h in cells labeled for 5 min with [35S]methionine in the presence of 80 μg/ml canavanine.

Table II

| Host* | Relevant genotype | β-Galactosidase* |
|-------|-------------------|------------------|
| SG20250 | Wild-type        | 50 units        |
| SG21118 | clpA319::kan | 178 units        |
| SG22007 | ΔclpP1::cat | 192 units        |
| SG22080 | ΔclpX1::kan | 49 units         |

* All strains are lysogenic for SB84, an imm21 clpA-lacZ translational fusion (21).

Table III

| Relevant genotype | Growth of λ in clp mutants |
|-------------------|---------------------------|
|                   | Protocol A* | Protocol B* |
| Wild-type         | Oam | Nam | Oam | Nam |
| clpP              | 16  | 6   | 0.5 | 9   |
| clpA              | 100 | 4   | 43  | 9   |
| clpX              | 15  | 3   | 0.8 | 34  |
| lon::Δtet         | 21  | 100 |      |      |

* Burst size was determined as the phage yield (titered on LE392) divided by the number of phage used for infection. Multiplicity of infection in all cases was 0.1 or lower.

In protocol A, SG20220 derivatives carrying an imm21plac-supF prophage were grown in the presence of isopropyl-1-2-thio-β-D-galactopyranoside (IPTG) as described under "Experimental Procedures," and infected with either imm434 Oam29 or imm434 Nam7Nam53 phage. Samples were taken and lysed with chloroform at 30-min intervals, beginning at 90 min after infection. The values shown in the table are for 90 min of infection. In the absence of IPTG, the burst size at 90 min was less than 1 for all strains.

In protocol B, SA1136 derivatives, which contain a temperature inducible cryptic O" prophage, were grown and induced for 10 min at 42 °C, and samples were then removed, infected with phage as described under "Experimental Procedures," and diluted into medium for growth with shaking at 30 °C. Phage growth was allowed to continue for 150 min. For cells kept at 30 °C throughout the experiment, the burst size for Oam phage in the SA1336 clp" host was 0.1, and for the clpP" mutant host was 0.8. The Nam phage gave a burst of 0.1 in SA1336 and 0.2 in the lon mutant host.

To estimate the effect of clp mutations on the amount of O protein accumulation in induced lysogens, the same cI857 Sam7 lysogens were grown at 30 °C and transferred to 42 °C for 30 and 60 min. Samples were analyzed for the accumulation of O protein by Western blot (Fig. 5B). In the induced

of β-galactosidase in cells carrying the fusion. Table II shows the results of β-galactosidase assays in isogenic strains carrying mutations in either clpX or another clp gene. The absence of any effect of the clpX mutation suggests that clpX activity is not necessary for degradation of this protein, a substrate of ClpA and ClpP.

O Protein-dependent Phage Growth—In the accompanying paper, Wojtkowiak et al. (17) have reported multiple protease activities capable of degrading X0 protein in vitro. They estimate that about 50% of the O protein degradation activity in crude extracts is due to ClpP/ClpX. We were interested in testing the contribution of ClpX to O protein turnover in vivo. We had previously found that mutations in lon or clpP did not affect O protein stability (4, 37).

We have used two different approaches to evaluate the role of clpP and clpX in O protein stability and function. In the first set of experiments, conditions under which O protein is likely to be limiting for growth were created, and the growth of an Oam phage was determined. The results of these experiments are shown in Table III. In the first set of conditions, a host carrying a suppressor tRNA gene under the control of a lac promoter was grown under inducing conditions, washed away from inducer, and used as the host for growth of the Oam phage. Growth of a wild-type phage (data not shown) or an Nam phage (Table III) was monitored as well, to control for other effects on phage growth. In efficiency of plating tests (data not shown) or burst size experiments (Table III), clpX and clpP mutations showed significantly better phage growth for the Oam phage than clpA mutants, wild-type cells, or lon mutant cells. The Nam phage, on the other hand, grew better in cells mutant in lon, the protease known to degrade N (37).

In the second phage growth test, cells carrying a temperature-inducible defective prophage which is O" and N" were induced for a short period of time, and then infected with the heteroimmune Oam or Nam phage, and allowed to continue growth at low temperature. Under these conditions, the O protein or N protein made from the cryptic prophage comple-
The results in this and the accompanying paper by Wojtkowiak et al. (17) indicate that the ClpXP protease has a substrate specificity distinct from that seen for the ClpAP protease.

In two other cases, evidence suggests that ClpX may be involved in ClpP-dependent degradation of specific substrates. A virulent Mu derivative is virulent and able to induce a resident prophage because the vir repressor is unstable and interacts with wild-type repressor to cause its degradation. That degradation was reported to be dependent on ClpP but not ClpA (16), and more recent results have shown that clpX mutations are at least as effective as clpP mutations in blocking the virulence of Muvir. A post-segregational killing mechanism encoded by phage PI also appears to depend on ClpXP activity. Cells which lose a vector carrying the pair of genes responsible for post-segregational killing normally die; hosts mutated in clpP or clpX but not clpA, clpB, or lon survive. This is presumably due to stabilization of the protective protein, Phd, in clpP or clpX mutant hosts.

Other results also indicate that ClpA is not necessary for degradation of specific substrates whose degradation is dependent on ClpP. Shapiro (41) has observed that the instability of Muclt lysogens on extended incubation of colonies is blocked by mutations in clpP, but to a much lesser extent by mutations in clpA. Damerau and St. John (42) have observed that degradation of proteins made during starvation after cells are returned to high carbon is decreased in clpP mutants but not in clpA or clpB mutants. Because most activities of ClpP appear to be dependent on an activator ATPase, it is reasonable to assume that an ATPase other than ClpA is involved in these degradation reactions; the required subunit could be ClpX. These sharp distinctions between substrates affected by clpA mutations and those affected by clpX mutations support the idea that the substrate specificity of the Clp proteases is dependent on the regulatory subunit and that only one such regulatory subunit is usually required for an active protease complex. Which of the available subunits ClpP associates with in vivo may depend on the availability of substrates as well as the availability of the regulatory subunit.

ClpX and its homologs (ClpX from A. vinelandii and the ClpY open reading frames from P. haemolyticus and B. subtilis) are significantly smaller than members of the ClpA family, which contain two highly conserved regions of approximately 200 amino acids in length, each containing a consensus sequence for an ATP nucleotide-binding site. The ClpX and ClpY proteins contain a single ATP-binding site consensus, with significant homology to those found in the ClpA/B/C family (Fig. 3). Beyond the ATP consensus sequences, ClpX, the ClpA/B/C family, and the putative ClpY proteins all share scattered homologies, most strikingly two clusters of amino acids in the COOH terminus, a region which is not highly conserved for the ClpA family except for these conserved patches (Ref. 7, Fig. 3). While the function of the COOH-terminal tail has not yet been determined, it seems possible that these conserved sequences are involved in interactions of these regulatory subunits with ClpP or ClpP-like subunits.

Unique to the two ClpX proteins is a cluster of cysteine residues, possibly a zinc binding motif, in the amino-terminal region; no function has yet been assigned to this structure for ClpX. The ClpX family, for which no in vivo or in vitro activity has yet been determined, lacks the cysteine cluster and is distinguished by a >100 amino acid insertion between parts A and B of the ATP consensus sequence. It is an 

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8 A. Toussaint, personal communication.
9 M. Yarmolinsky, personal communication.
intriguing possibility that a protein of the ClpY family might also exist in *E. coli* and might work with ClpP or a different protease to promote specific degradation of yet another set of substrates.

The occurrence of multiple ATPase subunits that can activate the proteolytic ClpP and direct it to different substrates has important implications for other ATP-dependent proteases. The eukaryotic ATP-dependent 26S protease contains the proteasome core associated with a number of other proteases that can modify its activity (43, 44). Among the associated proteases are one or more ATPases, which presumably carry out functions analogous to those performed by ClpA and ClpX for ClpP. Recently, Rechsteiner and co-workers (35) have reported that subunit 4 of the 26S protease, which has a single ATP-binding site consensus, is distantly related to ClpA. Subunit 4 is itself a member of a large family of ATPases which have been implicated in protein localization and gene regulation; these authors suggest that other members of this family may also be protease regulatory subunits. A second, 100-kDa subunit of the 26S ATP-dependent protease apparently has homology to the cdc48 subfamily of these ATPases (45). The Subunit 4 sequence reported by Dubiel et al. does not reveal any closer similarity between ClpX and subunit 4 than between ClpA and subunit 4. In particular, the motifs in the tail that are highly conserved between ClpX/Y and the ClpA/B/C family (Fig. 3) are not found in subunit 4 or its more closely related relatives. It will be interesting to see whether any of the other subunits of the 26S protease bear a closer resemblance to the Clp family subunits.

Unlike either ClpA or ClpB, the other member of the ClpA family found in *E. coli*, ClpX is synthesized as part of an operon with ClpP. Therefore, it shares with ClpP the ability to be induced as a heat shock protein. Our results indicate that a single message carrying both clpP and clpX is the primary transcript at both low and high temperatures. The 2-fold increase in the amount of message at high temperature is consistent with the 2-fold increase in ClpP protein synthesis reported by Kroh and Simon (10). Since the ClpX homolog in *A. vinelandii* is in the middle of an operon of *nil* (nitrogen fixation) genes (31), the location next to ClpP is apparently not a conserved feature of this protein.

**O Protein Stability and λ Replication**—λ O protein, like a number of other important regulatory proteins in bacteriophage λ, is rapidly turned over in *vivo*. We show here that this in *vivo* turnover is dependent on the activity of a novel protease composed of ClpP, the protease subunit of the Clp ATP-dependent protease (8) and ClpX, a new regulatory subunit capable of working with ClpP. The rapid turnover of λ O protein has been assumed until recently to reflect its role as a rate limiting step for lambda replication. Recent work by Taylor and co-workers (39, 46), in which they observed the accumulation of a stable subpopulation of O protein, apparently in the replication complex, suggests that O protein instability may not limit replication after all. Here we find that stabilizing O protein by mutations in clpX or clpP has no discernible effect on the burst size of an induced Cl857 Sam7 phage. However, under conditions where the availability of O protein is limited by either low level suppression of phage carrying Oam29 or by complementation of an Oam phage by transient induction of a cryptic prophage, it is clear that clpX or clpP mutants allow significant increases in phage yield (Table III). Therefore, it would appear that the rapid turnover of O protein limits replication activity only under suboptimal conditions, when O protein becomes limiting.

Engelberg-Kulka and co-workers (47) found that expression of λ protein RexB from a plasmid stabilizes O protein made from either the same or a compatible plasmid. While RexB is made in small amounts in lysogens, its synthesis increases from the pil promoter when replication of λ increases (48). They suggest that this increase in RexB synthesis results in O protein stabilization, which in turn allows further increases in replication. As we have noted here, at least under induction conditions, O protein stability does not limit burst size, so if this circuit exists it must provide an additional safeguard for protection of O protein under special conditions.

λ utilizes a number of unstable proteins at important stages in its life cycle; it is striking that thus far, each of these proteins is subject to proteolysis by a different system. Thus, CI degradation depends on the products of the hfl locus (49, 50). AN protein is subject to Lon degradation (37), CI degradation, part of the SOS response, is dependent on RecA, and sequences within the Cl protein (51). Here we show that O protein is turned over by a novel Clp variant, the Clp XP protease. Aαs protein is also known to be functionally unstable (52) and is not stabilized in lon mutants; its degradation may be dependent on yet another *E. coli* protease. Whether the use of these different proteases is simply accidental or reflects some additional regulatory network for λ is not yet clear.

**Acknowledgments**—We thank M. Zylicz for communicating both the results on the *in vitro* degradation of O protein and the NH2-terminal sequence of LopC to us; these results led us to carry out the experiments described in this paper. We thank S. Highlander, G. Weinstock, P. F. Slack, and L. Spenesheim for providing the information on unpublished sequences for the *P. haemolytica* and *B. subtilis* proteases, and A. Toussaint and M. Yarmolinsky for communicating unpublished information on ClpX targets. We are grateful to S. Wickner and R. McMacken for providing O protein, antibody, and plasmids used in this work. We thank S. K. Singh for constructing the pLClpX plasmid used for purifying ClpX for antibody isolation.

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