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Biochemistry

Protein degradation in *Escherichia coli*: The *lon* gene controls the stability of sulA protein

(cell division/SOS regulation/bacteriophage λ)

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ABSTRACT **_Escherichia coli lon_ mutants are defective in the ATP-dependent proteolysis of abnormal proteins. The mutants are also sensitive to ultraviolet light (UV) in that septation is inhibited after exposure to UV. _sulA_ mutations, isolated as suppressors of UV sensitivity unlinked to _lon_, do not affect proteolysis but allow septation to occur after DNA damage. We have confirmed the hypothesis that the product of the _sulA_ gene is degraded by _lon_ proteolysis. If sulA (the product of _sulA_) is a UV-inducible division inhibitor, as suggested by a variety of experiments, lon (the product of _lon_) may regulate cell division by regulating the half-life of sulA. We cloned the _sulA_ gene in a bacteriophage λ vector from a plasmid carrying the _ompA_ region of _E. coli_. An 18-kilodalton polypeptide was identified as the product of the _sulA_ gene. Pulse–chase labeling demonstrated that the half-life of the sulA protein is 1.2 min in _lon_$^+$ cells and 19 min in _lon_$^-$ cells. This work demonstrates that lon proteolysis affects the stability of a native _E. coli_ protein.**

The *lon* mutations of *Escherichia coli* affect a variety of physiological processes. *lon* mutants have decreased ability to degrade abnormal proteins (1–4), overproduce capsular polysaccharide (5), and are defective for lysogeny of bacteriophages λ and P1 (6, 7). They are also sensitive to DNA-damaging agents, such as ultraviolet light (UV) or methyl methanesulfonate (MeMes) (8). This UV sensitivity seems to result from an exaggeration of the normal inhibition of *E. coli* cell division after DNA damage (9). *lon* mutants do not recover from this division arrest and are therefore UV and MeMes sensitive.

Mutations identified in *lon* strains as suppressors of sensitivity to UV or MeMes map at two loci, *sulA* and *sulB*. These suppressors block the lethal filamentation seen in *lon* strains but do not affect other *lon* phenotypes (10–14). *sulA* and *sulB* also do not affect the cell's UV repair system or its induction. George and her co-workers (15) proposed that a division inhibitor, induced after UV irradiation, might be a target for the *lon* protease. The properties of *sulA* mutations suggest that the *sulA* gene product may be an inhibitor of septation, induced after DNA damage (14, 16). *sulB* mutations, which are rare relative to *sulA*, are dominant and may be in a gene encoding an essential cell division function that is a target for the *sulA* inhibitor. If sulA, the product of the *sulA* gene, were unstable and inactivated by lon proteolysis, its persistence in *lon* mutants might lead to excessive filamentation (13–15).

Recently the product of the *lon* gene has been identified as an ATP-dependent protease (17, 18), suggesting that the primary defect of the *lon* mutants may be the defect in proteolysis. If so, *lon* must exert its pleiotropic effects on cellular physiology by affecting the half-life of proteins involved in such processes as capsular polysaccharide synthesis or septation inhibition. Thus far, however, the lon protease has only been demonstrated to affect the degradation of abnormal proteins and the bacteriophage λ N protein *in vivo* (19). *In vitro*, it digests artificial substrates such as casein and hemoglobin (18). In the work described here we have asked directly if a protein implicated in the control of one set of *lon*-perturbed functions is a target for the lon protease.

We have cloned the *sulA* gene, identified the gene product, and examined the stability of sulA in *lon*$^+$ and *lon*$^-$ strains. We show evidence that sulA is degraded by lon proteolysis. Our results support the hypothesis of George *et al.* (15) and are sufficient to explain the UV sensitivity of *lon* strains.

MATERIALS AND METHODS

Bacteria and Phage Strains. The important bacterial and phage strains used are listed in Table 1. All strains not listed are from the National Institutes of Health strain collection. The procedure for P1 transduction has been described (14). Tetracycline-sensitive derivatives (Tet^s) of strains carrying transposon Tn*10* were isolated by the procedure of Maloy and Nunn (22). λ phages λimm21*c*I and λimmλ*c*Ib2 were used as *int*$^+$ helper phages to lysogenize λSM1 and λSM5, respectively, at the bacterial attachment site. The presence of the *sulA* and *ompA* mutations was determined by resistance to MeMes (3) and K3 phage (23), respectively.

Preparation of DNA. DNA of plasmid pTU100 was prepared according to the procedure of Clewell and Helinski (24). Closed circular DNA molecules were purified by a CsCl/ethidium bromide equilibrium density gradient centrifugation followed by dialysis against 10 mM Tris·HCl, pH 7.5/1 mM EDTA. High-titer phage stocks were prepared by the method of Yamamoto *et al.* (25). The phage were concentrated by polyethylene glycol precipitation and further concentrated and purified by CsCl equilibrium density gradient centrifugation. The purified phage were dialyzed against 10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. Phage DNA was extracted with phenol and dialyzed against 10 mM Tris·HCl, pH 7.5/1 mM EDTA.

Procedure for UV-Inducible Filamentation. UV treatment and preparation of samples for microscopy were carried out as described (14). Samples were fixed and kept in 2% (wt/vol) formaldehyde solution, collected, and resuspended in 10 mM $MgSO_4$ before layering on slides and were observed by phase-contrast microscopy in a Zeiss instrument.

***N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine Mutagenesis of *sulA* Transducing Phage.** An *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized lysate was prepared essentially by following the procedure of Adelberg *et al.* (26). N99 (F$^-$ *gal rpsL*) was

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Abbreviations: MeMes, methyl methanesulfonate; Tet^s, tetracycline sensitive; kb, kilobase(s); kDal, kilodalton(s).

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Table 1. Bacterial and bacteriophage strains

Strain name	Genotype	Source
HU100(pTU100)	F⁻ *thi pyrD gltA galK trp recA ompA rpsL* (pTU100)	U. Henning (20)
SM8	F⁻ *his pyrD leu rpsL*	SG13107 (14); select Tet^s
SM32	F⁻ *his pyrD Δlon-100 gal rpsL*	P1 (*galE*::Tn*10*) to SG13009 (14); Tet^s
SM37	F⁻ *his leu Δlon-100 sulA366 gal rpsL*	P1 (*galE*::Tn*10*) to SG13082 (14); Tet^s
SM39	F⁻ *his pyrD sulB367 Δlon-100 gal rpsL*	P1 (*galE*::Tn*10*) to SG13083 (14); Tet^s
SM40	F⁻ *his leu Δlon-100 sulA366 gal trp*::Tn*10 supF*	P1 (*trp*::Tn*10 supF*) to SM37
λD69	*Bam1*° *srlΔ*(1, 2) *imm*21 *nin*5 *Hind6*°	(21)
λSM1	*ompA*⁺ *sulA*⁺ *Bam1*° *srlΔ*(1, 2) *int imm*21 *nin*5 *Hind6*°	This work; orientation 1
λSM2	Same as for λSM1; orientation 2	This work
λSM4	*Bam1*° *srlΔ*(1, 2) cI857 *nin*5	λD69 × λ*bio*11 *nin*5 cI857
λSM5	*ompA*⁺ *sulA*⁺ *Bam1*° *srlΔ*(1, 2) *int* cI857 *nin*5	λSM1 × λ*bio*11 *nin*5 cI857

grown to 2×10^8 cells per ml in TBMM medium (10 g of Bactotryptone per liter/86 mM NaCl, 10 mM $MgCl_2$/0.2% maltose) at 37°C, infected with λSM5 at a multiplicity of infection of 0.5, and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10 μg/ml) for 15 min at 37°C. Mutagen-treated infected cells were collected and resuspended in 2 vol of prewarmed L broth (15 g of tryptone, 5 g of NaCl, and 5 g of yeast extract per liter), distributed in four aliquots, and incubated for 90 min at 37°C. The resultant lysate was treated with $CHCl_3$ and centrifuged to remove debris. Under these conditions, the frequency of clear mutations was 1–2% and the viability of the noninfected cells was 30–50%.

Protein Synthesis in UV-Irradiated Bacteria. The procedure was modified from that of Jaskunas *et al.* (27). Unless otherwise indicated, the cells were grown and treated in minimal medium M56 (28) supplemented with 0.2% maltose, uracil at 15 μg/ml, and 18 amino acids (excluding methionine) each at 25 μg/ml. At an OD_{650} of 0.3, the cells were concentrated 5-fold in medium supplemented with histidine and leucine (required amino acids, both at 25 μg/ml) instead of the 18 amino acids, irradiated with an incident dose of 1,000 J/m^2 at 254 nm, diluted 1:5 in the medium containing the 18 amino acids, and incubated at 37°C for 10 min in the dark. The cells were collected, resuspended in the medium containing 10 mM $MgCl_2$ at a concentration of 2×10^9 cells per ml, infected with phage at a multiplicity of infection of 5, and left on ice for 20 min to allow phage to adsorb. The cells were incubated in a 37°C water bath for 2 min, then diluted 1:20 with prewarmed medium and incubated at 37°C for an additional 5 min. Labeling of the proteins was started by adding [^{35}S]methionine (20 μCi/ml, 1,000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) to the samples and stopped after 5-min incubation by adding ¼ vol of 50% trichloroacetic acid/0.5% unlabeled methionine. In some cases UV-treated cells were labeled with a ^{14}C-labeled amino acid mixture (New England Nuclear, 10 μCi/ml, 55 Ci/mol of carbon) in medium supplemented with required amino acids. The labeled cells were collected by centrifugation, washed with cold acetone, dried, and suspended in 40 μl of sample buffer for $NaDodSO_4$/polyacrylamide gel electrophoresis. The gel electrophoresis was carried out by the procedure of King and Laemmli (29) except that the gels were 7.5–20% linear gradient gels. In most cases gels were fixed in 50% trichloroacetic acid for 30 min and then soaked in 25% methanol/10% acetic acid (vol/vol) for at least 1 hr. Some gels were stained in 50% trichloroacetic acid/2.5% Coomassie brilliant blue for 30 min and destained in 25% methanol/10% acetic acid. The labeled protein bands were visualized by fluorography (30). To estimate the molecular weight of the labeled proteins, the following proteins were used as standards: phosphorylase b (subunit molecular weight, 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), ferritin (18,500), and α-lactalbumin (14,400).

For quantitative data, a portion of the gel corresponding to the protein band of interest was cut out and soaked in 0.1 ml of distilled water at room temperature for 2 hr. Then 0.3 ml of Protosol and 4 ml of Econofluor (both from New England Nuclear) were added, and the mixture was incubated in a 37°C water bath overnight before radioactivity was measured.

RESULTS

Cloning of the *sulA* Gene. To allow cloning of the *sulA* gene, we have looked for closely linked markers to use for selection. The *sulA* gene cotransduces with *pyrD* at a frequency of 50–65% (3, 10, 13); *ompA*, coding for one of the major outer membrane proteins of *E. coli*, has a similar cotransduction pattern, and lies on the same side of *pyrD* as *sulA* (10). In P1 transduction experiments, *sulA* and *ompA* were very closely linked to each other (data not shown).

The *ompA* gene has been cloned in low-copy-number plasmids (20, 31). A 1.78-kilobase (kb) *Bam*HI fragment that carries the sequence for the amino terminus of the *ompA* protein and is sufficient to encode ompA complementing activity was taken from the low-copy-number plasmid pTU100 and cloned in λD69 by inserting into the *Bam*HI site in the λ *int* gene (Fig. 1). Phages were isolated with the *Bam*HI fragment in either orientation; both phages λSM1 and λSM2 complemented *ompA*.

A λ*sulA*⁺ phage should restore MeMes sensitivity to a *lon sulA* host. λSM1 lysogens of the *lon sulA* strain SM37 were isolated, with the prophage located either at *attB* or near the *ompA* locus. λSM2 lysogenizes poorly in the *lon sulA* strain, although lysogens can be isolated in a *proC lon*⁺ *sulA* strain and the *lon* mutation subsequently can be introduced by P1 transduction, selecting for proline independence and screening for mucoidy. We believe the difficulty in establishing lysogens is due to overproduction of sulA (see *Discussion*). Both λSM1 and λSM2 lysogens of *lon sulA* were sensitive to MeMes, and λSM2 lysogens were slightly filamentous even in the absence of MeMes. λ carrying the 1.78-kb fragment also complement *sulA* for UV-inducible filamentation; a *lon sulA*(λ*sulA*⁺) lysogen forms long filaments after UV treatment, as does a *lon sul*⁺ strain. This effect is specific to *sulA*; the transducing phage does not cause filamentation in *lon*⁺ or *lon sulB* strains after UV treatment. These results indicate that the 1.78-kb fragment carried by λ*sulA*⁺ contains the *sulA* gene and that this gene is functional in a lysogen.

Isolation of Phages Carrying *sulA* Mutations. On *lon* bacterial lawns, plaques of an *imm*λcI857 derivative of λSM1, λSM5*sulA*⁺, are clear, whereas they are turbid on *lon*⁺ lawns. The simplest explanation for this phenomenon is that multiple

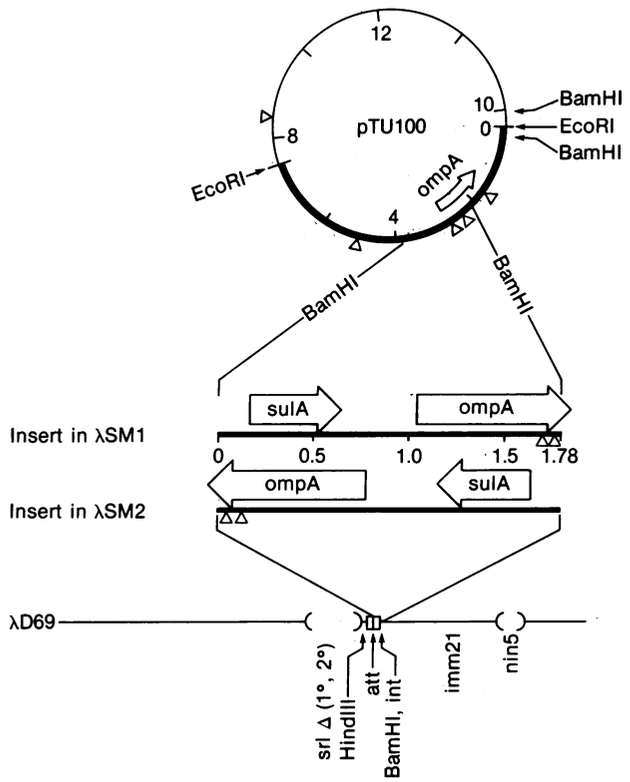

FIG. 1. Construction of λSM1 and λSM2. A 1.78-kb *Bam*HI fragment of pTU100 was inserted into the *Bam*HI site of the λD69 vector phage (21) and packaged *in vitro* according to the procedure of Enquist and Sternberg (32). Phages with inserts in the *int* gene were detected by the red plaque test (33). The orientation of the insert was determined by digestion with *Hin*dIII and *Pvu* II (the sites are indicated as triangles). The restriction map of pTU100 and schematic diagram of the reading frames for the ompA and sulA proteins are cited from Henning *et al.* (20) and Beck and Bremer (34), respectively. In the Beck and Bremer sequence of this region, sulA protein is indicated as a hypothetical 17-kilodalton (kDal) protein.

infection with sulA$^+$ transducing phage leads to enough sulA expression to kill lysogens growing in the plaque. Consistent with this explanation is the observation that in a lon sulB strain, in which sulA may not be able to act, λSM5 plaques are not as clear as on *lon* or *lon sulA* strains. To isolate a *sulA* mutation on the λSM5 phage, the *lon sulA* strain SM37 was lysogenized with λSM5. From this MeMes-sensitive lysogen, MeMes-resistant colonies were selected. Phage induced from the MeMes-resistant lysogens no longer complement *sulA*, still complement *ompA*, and form turbid plaques on *lon* hosts.

The difference in plaque morphology for phage carrying *sulA*$^+$ and *sulA*$^-$ was used to screen for additional *sulA* mutants on the phage. λSM5 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 50 turbid-plaque-forming phage were isolated from 2,800 plaques on a *lon* bacterial lawn. Thirty-five of these were *sulA* mutants as judged by their failure to complement *sulA* for MeMes sensitivity. One mutant, λ*sulA46*, acts as if it has an amber mutation: lysogens of a *lon sulA supF* strain form filaments on MeMes agar, whereas lysogens of a *sup*$^+$ strain do not.

Twelve λ*sulA* mutants, including *sulA46*, were shown to still be *ompA*$^+$. Therefore, *ompA* and *sulA* act as separate genes.

Identification of the Gene Product. The proteins coded for by the 1.78-kb fragment carried by λSM5 were determined by infection of UV-irradiated cells followed by labeling with [^{35}S]methionine (Fig. 2) or [^{14}C]leucine (data not shown). Compared with infection with the λ parental vector, three additional protein bands with the molecular masses of 27, 24, and 18 kDal were observed after infection with λSM5. The 18-kDal protein is apparently the product of the *sulA* gene. The 18-kDal protein band is missing after infection with 4 of 11 mutant phages, including *sulA46* (Fig. 2*A*), whereas the 27- and 24-kDal proteins are present after infection with all 11 mutants. λSM5*sulA46*, carrying an amber mutation in *sulA*, produced the 18-kDal protein in *supF* host cells (Fig. 2*B*). The 27- and 24-kDal protein bands are consistent with the size for a truncated ompA protein (24 kDal) and its unprocessed precursor (pro-ompA, 27 kDal) (34).

Stability of the sulA Protein. Is the sulA protein degraded by a *lon*$^+$-dependent process? The stability of the sulA protein was assayed in *lon*$^+$ and *lon*$^-$ strains by pulse–chase labeling of the proteins synthesized after infection of UV-irradiated hosts with the *sulA* transducing phage. Host cells were treated with UV, infected with λSM5, pulse labeled with [^{35}S]methionine, and chased with an excess of unlabeled methionine. Samples

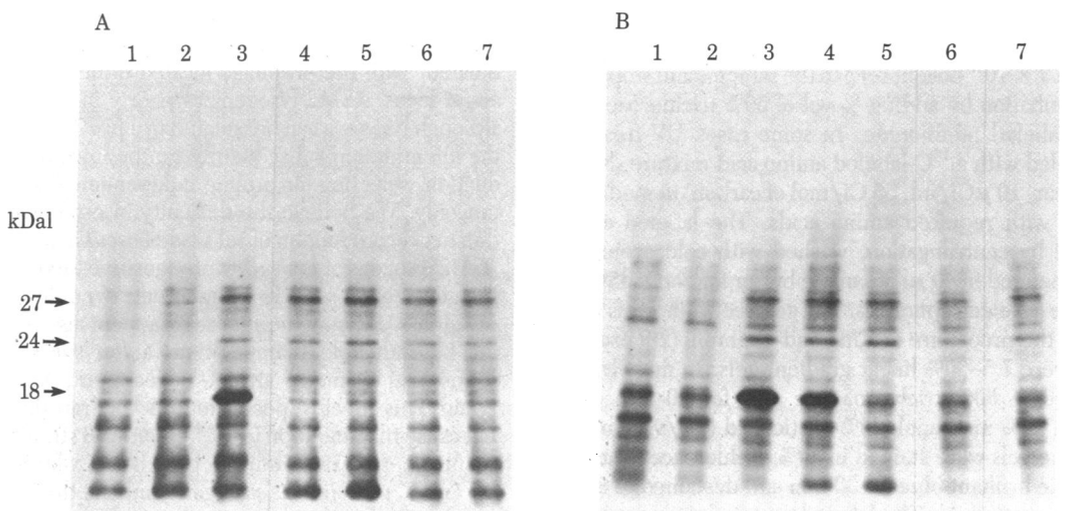

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of proteins synthesized after infection of UV-irradiated cells. Cells were *sup*$^+$ (SM37) in *A* and *supF* (SM40) in *B*, both lysogenic for λ*ind*. Cells were infected after UV irradiation as follows: lane 1, no infection; lane 2, vector, λSM4; lane 3, wild-type phage, λSM5*sulA*$^+$; lane 4, λSM5*sulA46*; lane 5, λSM5*sulA41*; lane 6, λSM5*sulA24*; lane 7, λSM5*sulA11*. The arrows indicate the position of the proteins specified by the 1.78-kb insert carried by λSM5*sulA*$^+$.

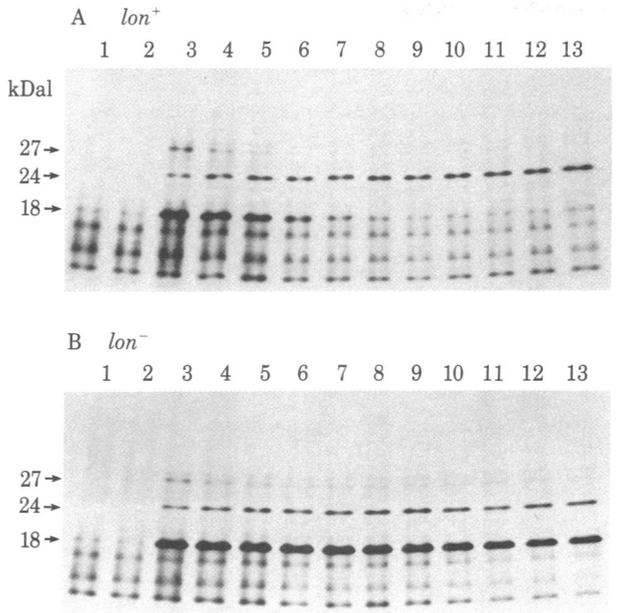

FIG. 3. Pulse-chase labeling of sulA in *lon*$^+$ and *lon*$^-$ cells. Cells were UV irradiated, and infected with λSM5, as for Fig. 2. Infected cells were pulse labeled with [^{35}S]methionine for 1 min and chased with 10^4-fold excess unlabeled methionine. Cells were *lon*$^+$ (SM8) in *A*, *lon*$^-$ (SM32) in *B*; both are lysogenic for λ*ind*. Controls are lane 1, no infection and, lane 2, infection with vector phage, λSM4, and sampled at 0 min of chase. Lanes 3–13, cells were labeled for 1 min (lane 3) and chased for 1 min (lane 4), 2 min (lane 5), 3 min (lane 6), 5 min (lane 7), 7.5 min (lane 8), 10 min (lane 9), 12.5 min (lane 10), 15 min (lane 11), 17.5 min (lane 12), and 20 min (lane 13).

were removed at various times after the pulse labeling and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. As shown in Figs. 3 and 4, the sulA protein has a half-life of 1.2 min in *lon*$^+$ cells and 19 min in *lon*$^-$ cells. Among the *E. coli* proteins coded for by λSM5, lon protease is specific for sulA; no decay

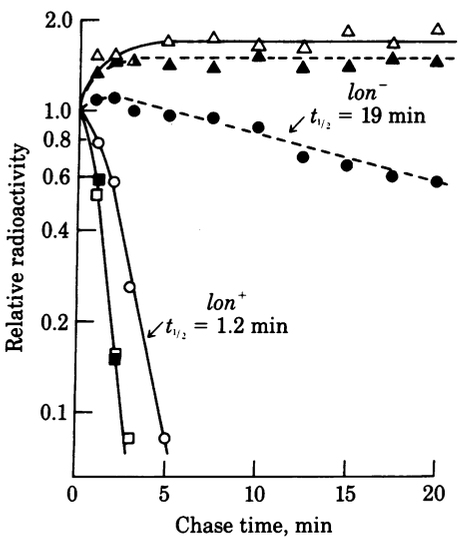

FIG. 4. Stability of sulA in *lon*$^+$ and *lon*$^-$ cells. The pulse–chase-labeled bands of the sulA, ompA, and pro-ompA proteins were cut out from the gels shown in Fig. 3, and the radioactivity in the bands was measured. The radioactivity remaining in the band is presented as relative to that found at 0 min of chase. Open symbols, bands from infection of *lon*$^+$ strain; closed symbols, bands from infection of *lon*$^-$ strain. △ and ▲, 24-kDal (ompA) band; □ and ■, 27-kDal (pro-ompA); ○ and ●, 18-kDal (sulA) band.

of the ompA protein was detected in either *lon*$^+$ or *lon*$^-$ cells. The half-life of the pro-ompA protein, 0.3 min, was the same in *lon*$^+$ and *lon*$^-$ cells. Similar experiments were carried out in a *lon sulB* strain; the *sulB* mutation has no effect on the stability of the sulA protein (data not shown).

DISCUSSION

Cell division in *E. coli* is normally coupled to DNA replication, so that anucleate cells are rare. DNA damage leads to inhibition of septation while cell mass increases, resulting in formation of nonseptated filaments. As DNA damage is repaired, normal cell division resumes. In *lon* cells, however, an exaggerated and lethal continuation of filamentation occurs (9).

sulA has been implicated in this process because mutations in *lon* cells that block lethal filamentation map in *sulA*, or, less commonly, in *sulB*. No other aspect of the SOS induced DNA repair system is apparently affected by the *sulA* and *sulB* mutations. George and her co-workers (15) postulated that a septation inhibitor coded for by one of the *sul* genes might be induced in response to DNA damage and that its persistence might be regulated by *lon* through proteolysis. The recent finding that *sulA* is transcriptionally induced after DNA damage (16) makes sulA the best candidate for such a division inhibitor.

We have cloned the *sulA* gene on a 1.78-kb *Bam*HI fragment, in a λ vector. Lysogens of λ carrying the fragment in either orientation complement *sulA*, suggesting that the fragment codes for the entire *sulA* gene, including its promoter. An amber mutation in *sulA*, *sulA46*, allows identification of the gene product as a protein with a molecular mass of 17–18 kDal. The fragment also encodes the amino terminus of the ompA protein, but our mutant analysis indicates that the two proteins are coded for by separate genes. Beck and Bremer (34), studying *ompA*, determined the DNA sequence of the whole *Bam*HI fragment and noted an open translational reading frame that could encode an 18-kDal protein. Because the sizes of the sulA protein and the hypothetical protein are identical, and no other open reading frame found on the fragment could encode a polypeptide with molecular weight near this, we propose that the open reading frame codes for the sulA protein.

The class of DNA-damage-inducible genes shares the property of being under control by *lexA* and *recA* as part of the SOS system (16, 35). For a number of these genes, a *lexA* binding site has been found in the promoter region (36–38). Upstream of the open reading frame for the *sulA* gene there are three potential promoter sequences. In addition, we have found a region in the Beck and Bremer sequence (at base pairs 130–149) highly homologous with the *lexA* binding site regions identified in the *recA*, *lexA*, and *uvrB* genes. Our potential *lexA* binding site shares 18/20 bases of homology with the lexA binding site of the *recA* gene. This region overlaps the −10 regions of two of the three promoters. The finding of this site is consistent with the finding by Huisman and D'Ari (16) that *sulA–lacZ* operon fusions act genetically as if they are repressed by *lexA*.

Huisman and D'Ari also found, in their *sulA–lacZ* fusion experiments, that *lon* does not transcriptionally regulate *sulA*. We found that *lon* regulates activity of *sulA* by degradation of the sulA protein. We have demonstrated that sulA is unstable in *lon*$^+$ cells, disappearing with a half-life of 1.2 min. In *lon*$^-$ cells, however, the half-life is 19 min. Our data are sufficient to explain the filamentation phenotype of *lon* mutants: if sulA is a division inhibitor and is synthesized for a short time after DNA damage, its persistence in *lon* cells would lead to extended inhibition of septation and therefore lethal filamentation. In *lon*$^+$ cells, the rapid decay of sulA would ensure a return to normal septation as soon as new sulA synthesis is shut off.

If sulA product is the only inducible function necessary for inhibition of septation, any mechanism that leads to its persistence would be sufficient for filamentation. Lysogens of λSM2 carry *sulA* downstream from the λ promoters p_L and p_{int}, in the proper orientation to be expressed from p_{int}. These strains filament slightly even in the absence of DNA-damage-inducing treatment in *lon* strains. Thus if p_{int} is expressing *sulA* at a low constitutive level (39), it may be sufficient to cause filamentation of *lon* host cells without any necessary induction of other SOS functions.

Thus we have demonstrated that lon degrades native *E. coli* proteins, as well as nonsense fragments (3) and a variety of denatured proteins (18). Among a number of unstable λ proteins examined, only the positive regulatory protein N was stabilized by lon (19). The half-lives of neither the pro-ompA protein nor the stable ompA protein are affected by the *lon* system. We conclude that lon is not generally responsible for the processing of membrane proteins. This is in contrast to the suggestion of Gayda *et al.* (40), who have proposed that the processing of the precursor of an outer membrane protein is regulated by lon. The other phenotypes of *lon* such as the overproduction of capsular polysaccharide in *lon* cells have not yet been satisfactorily explained. If the mechanism of *lon* control of this phenomenon is similar to its control of cell division, we would predict that a positive regulator of polysaccharide synthesis should exist, and its stability would be regulated by lon proteolysis.

The recA protease has an even more striking specificity than lon, cleaving only a handful of phage repressors and the cellular repressor lexA. The half-life of the λ control protein cII may be regulated by the cellular *hf1* function (41). Therefore, *E. coli* may utilize a set of proteases for fine-tuning and timing regulation of important cellular processes.

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