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Isolation and Characterization of *lon* Mutants in *Salmonella typhimurium*

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In this paper we report the isolation and characterization of *lon* mutants in *Salmonella typhimurium*. The mutants were isolated by using positive selection by chlorpromazine resistance. The physiological and biochemical properties of the *lon* mutants in *S. typhimurium* are very similar to those of *Escherichia coli lon* mutants. Mutants altered at this locus contain little or no activity of the ATP-dependent protease La and show a number of pleiotropic phenotypes, including increased production of capsular polysaccharides, increased sensitivity to UV light and other DNA-damaging agents, and a decreased ability to degrade abnormal proteins.

The *lon* locus of *Escherichia coli* has been extensively characterized both genetically and biochemically (7, 8, 12, 14, 18, 28). The product of the *lon* gene is an ATP-dependent protease, also called protease La (5, 7), whose function requires concomitant ATP hydrolysis (18, 28). Current evidence suggests that protease La acts in vivo to degrade highly abnormal proteins that may arise through nonsense mutations or incorporation of amino acid analogs or puromycin into proteins (10, 18, 28). In addition, there is now clear evidence that protease La is involved in the degradation of certain normal proteins (9, 11, 26) such as the *sulA* gene product, which is believed to be an inhibitor of cell division induced during an SOS response (15). Mizusawa and Gottesman (20) have recently shown that the *sulA* protein is very rapidly degraded in wild-type cells, but this process is reduced approximately 15-fold in *lon* cells. It appears likely that protease La is responsible for degrading the *sulA* protein and thereby restores the cell's ability to undergo cell division. Protease La probably has regulatory roles in addition to its role in the SOS response. For example, *lon* mutants also show derepression of at least five biosynthetic genes for capsular polysaccharide (19, 26).

E. coli lon mutants produce a defective ATP-dependent protease and show a complex phenotype, presumably because of the many cellular processes involving short-lived polypeptides. Strains with mutations in the *lon* locus have a decreased ability to degrade various abnormal proteins, especially nonsense fragments or temperature-sensitive proteins (1, 3, 11–13, 20). Due to their overproduction of capsular polysaccharides, *lon* cells on agar plates form mucoid colonies which are easy to distinguish morphologically from wild-type colonies (17). In addition, *lon* strains are sensitive to UV light and other DNA-damaging agents (14), apparently because of an inability to recover from the arrest of cell division brought on by an SOS induction.

Very little is known about the genetics and function of the *lon* gene in *Salmonella typhimurium*. In the course of work on SOS induction in *S. typhimurium*, we needed to test various effects of *lon* mutations. Therefore, we have isolated and characterized several such mutants. We used a positive selection method, reported by Molnar et al., that is based upon the resistance of *lon* cells to chlorpromazine (21). In this paper we report isolation and characterization of *lon* mutants of *S. typhimurium*.

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MATERIALS AND METHODS

Bacterial strains. All strains used in this study and their sources are listed in Table 1. All *S. typhimurium* strains are derived from LT2. Strains CGSC5218 is an original isolate of *E. coli*.

Media. The E medium of Vogel and Bonner (27) supplemented with 0.2% glucose was used as minimal medium. Alternative carbon sources were added at 0.2% to E medium lacking citrate (24). Difco Laboratories nutrient broth (8 g/liter) with NaCl added (5 g/liter) was used as a rich medium. Difco Bacto-agar was added to a final concentration of 1.5% for solid media. To purify transductants, single colonies were isolated on the green indicator agar of Chan et al. (4).

The following additives were included in media as needed (final concentrations given): tetracycline (25 μg/ml in rich media, 15 μg/ml in minimal medium), chlorpromazine (75 μg/ml), nitrofurantoin (3 μg/ml), and puromycin (150 μg/ml).

Transduction methods. The high-frequency generalized transducing bacteriophage P22 mutant (HT105/1, *int-201*) (25) was used for all transductional crosses. Recipient cells (10^8) and transducing phage (10^8 to 10^9 PFU) were spread directly on selective plates. Transductants were purified and verified to be phage free by streaking on nonselective green indicator plates (4). Phage-free colonies were checked for phage sensitivity by cross-streaking with phage P22 H5 (a clear-plaque mutant).

During the tranductions involving the mucoid *lon* strains, it became apparent that phage sensitivity could not always be determined in the standard way. Generally P22-sensitive strains of *S. typhimurium* form light colonies on the green indicator medium used for purification (4). The mucoid *lon* strains remain a distinctive green color on the indicator medium and show no lysis by P22 when tested by cross-streaking on solid media. These *lon* strains retain a normal ability to be transduced and can support growth of high-titer phage lysates when the cells and phage are mixed in liquid. Apparently, in liquid cultures, there is less accumulation of capsular polysaccharide on the cell surfaces, and phage adsorption is possible. Spontaneous nonmucoid revertants

TABLE 1. List of strains

Strain	Genotype	Source
TR6543	*lon-71*	This study
TR6643	*lon-72*	This study
TR6644	*lon-73*	This study
TR6645	*lon-74*	This study
TR6646	*lon-75*	This study
TR6647	*lon-76*	This study
TR6648	*lon-77*	This study
TT8024	*lon-71 zaj-1034*::Tn*10*	This study
TT8025	*lon*$^+$ *zaj-1034*::Tn*10*	This study
CGSC5218	*metB1 relA1* /F′$_{13}$ (deletion of chromosome corresponding to F′)	*E. coli* stock center
SG1406	*E. coli*/F′$_{13}$ *lon-100* Tet^r	S. Gottesman
SG1410	*E. coli*/F′$_{13}$ Δ*lon-2* Tet^r	S. Gottesman

(which still contain the *lon* mutation) show normal behavior on green indicator medium and in cross-streaking tests.

Mutant isolation. Mucoid mutants were isolated by a modification of the procedure of Molnar et al. (21). Cultures of *S. typhimurium* LT2 were grown overnight in nutrient broth. Approximately 10^8 cells of an overnight culture were added to 5 ml nutrient broth containing 75 g of chlorpromazine per ml and incubated at 37°C with shaking for 4 to 48 h. Cultures which were turbid after this time were diluted 10^4- and 10^6-fold and plated on minimal agar plates. Mucoid colonies were characterized further.

Degradation of puromycyl peptides. Strains to be tested for degradation of puromycin-containing polypeptides were grown in glucose minimal medium (M9) supplemented with tetracycline. During logarithmic growth, puromycin (150 μg/ml) was added, and incubation was continued for 15 min; then [4,5-^{3}H]leucine (1 μCi/ml; New England Nuclear Corp.) was added for 5 min. After filtration the cells were washed and suspended in growth medium containing excess nonradioactive leucine (1 mg/ml) to prevent reincorporation of [^{3}H]leucine released by proteolysis. The appearance of acid-soluble radioactivity was measured as previously described (7).

Enzyme assays. Protease was partially purified from crude cell extracts by phosphocellulose chromatography. These preparations were assayed for their ability to hydrolyze [^{3}H]casein and glutaryl-Ala-Ala-Phe-methoxynaphthylamine (-MNA) in both the presence and absence of ATP. This procedure has been described in detail elsewhere (10a, 28a).

RESULTS

Isolation of *lon* mutants. Independent cultures of *S. typhimurium* LT2 were grown in the presence of chlorpromazine (see Materials and Methods). After 36 h at 37°C, half of the cultures started in this way had not grown to full density. Of the cultures that did grow to full density, 60% produced only mucoid colonies, and 40% produced only morphologically normal colonies. The mucoid colonies were characterized, and their properties are described below.

UV-sensitivity of *S. typhimurium lon* mutants. Since *lon* mutants of *E. coli* are known to be sensitive to UV light and other DNA-damaging agents (14), the new mutants were checked for this property. Fifty colonies from each of the cultures described above were patched to a nutrient broth plate. These master plates were then replica printed to nutrient broth plates and irradiated with a UV dose known to completely kill a patch of *recA* cells. When screened in this way, 80% of the mucoid colonies were UV sensitive. None of the nonmucoid colonies appeared sensitive to UV light.

When these same mucoid colonies were tested on a glucose minimal plate they were not detectably sensitive to UV light. The phenomenon of post irradiation plating media affecting sensitivity to UV has been reported for *E. coli lon* mutants (14). The increased radiation sensitivity seen on nutrient broth plates is attributed to the presence of NaCl and amino acids. In addition, the mucopolysaccharide overproduction is more pronounced on media containing glucose such as our minimal medium; this excess polysaccharide may offer a shield against UV.

To define more clearly the radiation sensitivity of these strains, a killing curve was determined. Figure 1 shows the percent survival as a function of time of irradiation with one 15-W germicidal lamp at a distance of 28 in. (ca. 70 cm). The radiation dose was determined to be 0.4 J/m^2 per s by T4$_{v1}$ inactivation as descried by Wulff (29). The *lon* mutants of *S. typhimurium* have a UV sensitivity which is between that of *recA* and wild-type strains (Fig. 1).

In addition, we tested the sensitivity of the *lon* mutants to the DNA-damaging agent nitrofurantoin. At a concentration of 3 μg/ml in nutient broth plates, this agent prevented growth of the *lon-71* mutant (TR6543), whereas the growth of the parent strain LT2 was unaffected (Table 2).

Isolation of a Tn*10* insertion near *lon*. For characterization of *lon*, we isolated a Tn*10* insertion near the mutant locus. To accomplish this, phage P22 was grown on a pool of over 10,000 random Tn*10* insertions, and the lysate was used to transduce TR6543 (*lon-71*) to tetracycline resistance (Tet^r) on rich medium (16). The Tet^r transductants were screened for those that inherited a wild-type *lon*$^+$ allele by cotransduction. Only those Tet^r tansductants which were UV resistant and nonmucoid were saved. This double screen was done to

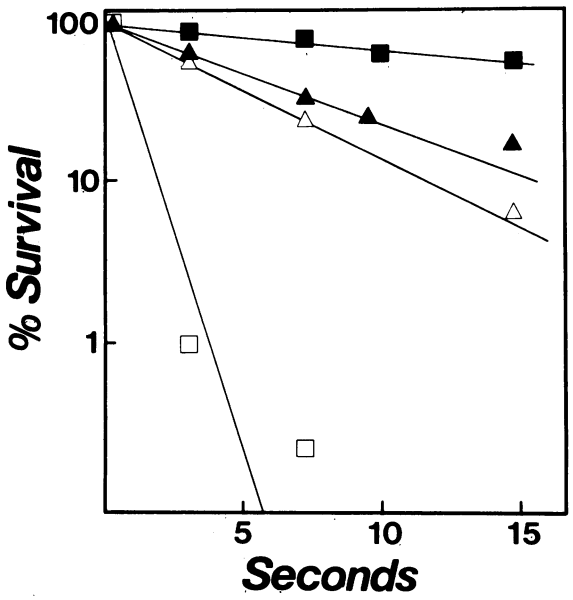

FIG. 1. Fraction of various strains which survive UV irradiation when plated on nutrient broth plates. Overnight cultures were diluted, plated on nutrient broth plates, and irradiated for various times at 28 in. from one 15-W germicidal bulb (0.4 J/m^2 per s). The percent survival was determined to be the fraction of cells surviving after irradiation compared with the nonirradiated control. Symbols: (□) *recA1*, (▲) TR6543 (*lon-71*), (△) TR6646 (*lon-75*), (■) LT2.

TABLE 2. Sensitivity to DNA-damaging agents

lon allele	Sensitivity to DNA-damaging agents[a]			
	+UV	−UV	+NF	−NF
lon$^+$	+	+	+	+
lon-71	−	+	−	+
lon-72	−	+	−	+
lon-73	−	+	−	+
lon-74	−	+	−	+
lon-75	−	+	−	+
lon-76	+/−	+	+	+
lon-77	−	+	−	+

[a] Patches of cells were tested for UV sensitivity by exposure to two 15-W germicidal bulbs at 28 in. Sensitivity to nitrofurantoin (NF) was scored by ability to grow on nutrient plates containing 3 μg of nitrofurantoin per ml.

avoid the possibility of picking up insertions in distant genes which suppress either the UV sensitivity (9) or the mucoid phenotype (23).

Several of these Tn*10* insertions were used as donors in transductional crosses with TR6543. By selecting resistance to tetracycline and scoring UV sensitivity in these crosses, it was possible to find an insertion, *zaj-1034*::Tn*10*, which is 39% linked to *lon-71*. This Tn*10* was tested for linkage to six other independent *lon* mutants which were isolated in the same way (Table 3).

In *E. coli* the closest marker to *lon* on the standard genetic map is the *proC* locus (2). Possible linkage of *lon* in *S. typhimurium* to *proC* was checked by using phage P22 grown on TR6543 as a donor to transduce *proC90* to prototrophy. Pro$^+$ transductants were scored for UV sensitivity. From this cross, no (0 of 100) Pro$^+$ transductants were found to be sensitive to UV light. In addition, linkage of *jaz-1034*::Tn*10* to *proC* was tested and found to be <0.1%. Thus no linkage of *lon* to *proC* was detected.

Complementation testing. In *E. coli*, *lon* maps at 10 min on the chromosome (2). To test whether the *lon* mutations isolated in *S. typhimurium* are complemented by the *E. coli* region carrying the *lon*$^+$ allele, we transferred the Lac$^+$ plasmid F′$_{13}$, which covers this region from *E. coli* into strain TR6543 (*lon*). After conjugation on lactose minimal plates, the Lac$^+$ colonies were streaked on nonselective media. These streaks produced a mixture of mucoid and nonmucoid colonies. When tested selectively, the nonmucoid colonies were Lac$^+$ (indicating the presence of F′$_{13}$), and the mucoid colonies were Lac$^-$. Colonies of each morphological type were again streaked out nonselectively; the streaks from the mucoid colonies remained mucoid, whereas the streaks from the nonmucoid clonies now contained some mucoid colonies. These results suggest that the mucoid colonies contain those cells which either never inherited or have subsequently lost F′$_{13}$. When UV sensitivity was tested, all mucoid colonies were UV sensitive and Lac$^-$, whereas all nonmucoid colonies were UV resistant and Lac$^+$. To show that the complementing ability of F′$_{13}$ was due to the *lon*$^+$ gene, we tested identical F′$_{13}$ plasmids carrying mutant *lon* alleles. These *lon* plasmids were transferred into *S. typhimurium lon* mutants selecting for inheritance of the F′$_{13}$ *lac*$^+$ genes on lactose minimal medium. The Lac$^+$ colonies were streaked selectively, and individual colonies were checked for UV sensitivity. None of the F′$_{13}$ *lon* plasmids showed complementation with the *S. typhimurium lon* mutants tested (*lon-73*, *lon-71*). The combined complementation results are shown in Table 4. We conclude that our mutants are complemented by the *lon*$^+$ gene of *E. coli*.

TABLE 3. Linkage of *zaj-1034*::Tn*10* to *lon* alleles[a]

Recipient	No. Tet^r	No. UV^r	% Linkage
TR6543	100	39	39
TR6644	92	68	74
TR6645	100	45	45
TR6646	100	75	75
TR6647	100	55	55
TR6648	96	72	75

[a] TT8025-*lon*$^+$ *zaj-1034*::Tn*10*, was used as a donor in a P22 transduction, selecting for tetracycline resistance. UV^r, UV resistance.

TABLE 4. Complementation with *E. coli* F′$_{13}$[a]

S. typhimurium allele	Genotype of *E. coli* plasmid	Growth on lactose	Colony morphology	Growth after UV
lon-71	None	−	M	−
lon-73	None	−	M	−
lon-71	F′$_{13}$ *lon*$^+$	+	NM	+
lon-73	F′$_{13}$ *lon*$^+$	+	NM	+
lon-71	F′$_{13}$ Δ*lon-2*	−	M	−
lon-73	F′$_{13}$ Δ*lon-2*	−	M	−
lon-71	F′$_{13}$ *lon-100*	−	M	−
lon-73	F′$_{13}$ *lon-100*	−	M	−

[a] M, Mucoid colonies; NM, nonmucoid colonies. The UV dose given was 10 J/m^2.

Protein degradation. Mutations of the *lon* locus in *E. coli* result in a reduced ability to degrade abnormal proteins (12, 17, 19, 21) as a consequence of the defect in protease La (7, 28). Strains TT8024 (*lon*) and TT8025 (*lon*$^+$) were compared in their ability to degrade incomplete proteins containing puromycin. Incorporation of puromycin into growing polypeptides leads to premature translational termination and release of the incomplete polypeptide from the ribosome. Such polypeptides are rapidly degraded in an ATP-dependent process (12). During logarithmic growth at 37°C in glucose minimal media, cells were exposed to puromycin (150 μg/ml) and [^{3}H]leucine. The degradation of polypeptides containing puromycin was then determined by measuring the percentage of the labeled polypeptide converted into acid-soluble material. *lon-71* has a defect in the

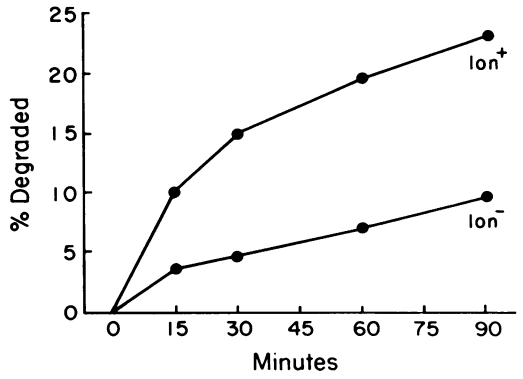

FIG. 2. Degradation of puromycyl peptides. Growing cultures of TT8024 (*lon-71*) and TT8025 (*lon*$^+$) were incubated with puromycin at 100 μg/ml for 15 min. [4,5-^{3}H]leucine was added for 5 min to label incomplete proteins. The cultures were washed and suspended in growth medium containing excess leucine. Samples were withdrawn at each time point, and acid-soluble radioactivity was measured.

TABLE 5. Protease La activity in *lon*$^+$ and *lon* strains of *S. typhimurium*[a]

Strain	[^{3}H] casein hydrolysis (μg/h)			Glutanyl-Ala-Ala-Phe-MNA hydrolysis (pmol/h)		
	−ATP	+ATP	Difference	−ATP	+ATP	Difference
lon	0.9	1.4	0.5	10	44	34
lon$^+$	0.5	6.8	6.3	38	2,450	2,412

[a] These assays were performed after partial purification of ATP-dependent protease activity from crude cell extracts by phosphocellulose chromatography as described previously (28).

breakdown of puromycyl peptides (Fig. 2). Strain TT8024 (*lon-71*) degrades these abnormal polypeptides about one-third as rapidly as TT8025, which contains the wild-type *lon*$^+$ locus. Thus, this defect is similar to that in *E. coli lon* mutants (7).

Proteolytic activity. Protease La, the product of the *lon* gene, was isolated from *S. typhimurium* strains TT8024 (*lon-71*) and TT8025 (*lon*$^+$) by phosphocellulose chromatography, as previously described for *E. coli* (28). In both *S. typhimurium* and *E. coli*, this treatment isolates from the bulk of all proteins an enzyme which degrades proteins and certain fluorogenic peptide substrates in an ATP-dependent reaction. The fraction isolated from the *lon* strain has very much less of this proteolytic activity than does the wild type (Table 5). It is noteworthy that the *lon* strains still showed some ATP-dependent casein-degradative activity, although it was only 10% of that in the wild-type strain. The protease La in *S. typhimurium* resembles that in *E. coli* in many features, e.g., its ATP dependence, its peptide preference, and its chromatographic behavior. Thus the defects in protease La caused by *lon* mutations in *S. typhimurium* closely resemble those in the analogous *E. coli* mutations.

DISCUSSION

By using chlorpromazine resistance as a selective treatment, we have obtained a number of *lon* mutants in *S. typhimurium*. The basis for the increased resistance of *lon* mutants to the drug chlorpromazine (21) is unclear. This compound binds tightly to several hydrophobic proteins and especially to calmodulin (6) and protein kinase C (22), neither of which is known to exist in *S. typhimurium* or *E. coli*.

In all the tests described above, *lon* mutants in *S. typhimurium* have properties which are indistinguishable from the published properties of the equivalent mutations in *E. coli*. The enzyme encoded by the *lon* gene has been purified and characterized from *S. typhimurium* (L. Waxman, A. S. Menon, and A. L. Goldberg, unpublished observations). Like the enzyme from *E. coli*, it is an ATP-dependent protease and hydrolyzes the same fluorometric model peptide glutaryl-Ala-Ala-Phe-MNA in an ATP-dependent reaction. The enzyme from *S. typhimurium* is sensitive to diisopropyl fluorophosphate (1 mM) and thus seems to be a serine protease with similar specificity to that from *E. coli*. In addition, it has a large multimeric size, as defined by chromatography on Sephacryl S-300, and is comprised of a single 94,000-dalton monomer, like protease La from *E. coli*. Finally, the *S. typhimurium* enzyme shows an inherent ATPase that is stimulated severalfold by protein substrates. Thus the products of the *lon* genes from *E. coli* and *S. typhimurium* are very similar in several respects including their chromatographic behavior, sizes, ATP binding, and mechanism of action (Menon, Waxman, and Goldberg, unpublished observations).

Certain alleles of *recA* in *S. typhimurium* become temperature-sensitive lethal alleles in the presence of these *lon* mutations. This lethality at high temperature in the presence of a *lon* mutation is also shown by *tif* alleles of *recA* in *E. coli* (9). Data describing this novel phenomenon will be reported in detail in a later publication.

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