GENETIC ANALYSIS AND PHENOTYPIC CHARACTERIZATION OF LON MUTANTS OF *ESCHERICHIA COLI* K-12

by

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INTRODUCTION

In the Gram-negative bacterium Escherichia coli K-12, mutants at a locus called lon (long filaments formed) have been isolated in which the coordination of cell division with DNA replication is defective following exposure to irradiation or treatments specifically inhibiting DNA synthesis (2, 78). X-ray or ultraviolet (UV) light irradiation results in the formation of long, non-septate filaments when cells are plated on rich medium (63,78, 91,178). These filaments can reach a maximal length of one hundred times that of normal cells (Lon^+) and eventually lyse (3,91,178). The extreme UV sensitivity of Lon^- strains is reflected in the formation and lysis of the filaments since the extent of killing is directly proportional to the number of filaments formed (2).

From genetic crosses, the lon mutation has been found to be indistinguishable from a mutation isolated by Markovitz (113) called capR (Regulator gene of capsular polysaccharide syntheses). CapR mutants appear as mucoid colonies when plated on minimal medium at either 30C or 37C due to the overproduction of capsular polysaccharide (colanic acid), in contrast to Lon^+ strains which are nonmucoid when plated on minimal medium (78). In addition, $CapR^-$ mutants exhibit the conditional cell division lesion observed in Lon^- mutants after exposure to UV light (41,78,115).

Other phenotypes have been described in Escherichia coli K-12 which are associated with the lon defect. Abnormal polypeptide degradation

(17,70,154) and decreased ability to form stable lysogens of either lambda (λ) or P1 bacteriophage, as well as maintenance of other extrachromosomal genetic elements (52,165), are also characteristic phenotypes of Lon$^-$ strains. Furthermore, Lon$^-$ mutants that are also thymine auxotrophs, exhibit enhanced sensitivity to growth conditions in which thymine is lacking (thymineless death) (178).

The apparent pleiotropic nature of the lon (capR) mutation suggested to some investigators a possible regulatory role of the lon gene product in a complex system of control, and several hypothetical models have been proposed (36,60,62,63,67,69,87,176,181) in an attempt to provide a molecular description of the possible pathways by means of which functionally distinct phenotypes could be controlled by the lon locus. Genetic studies have been made to describe precisely the nature of the lon locus (20,42), but there appears to be no consensus about how many cistrons are encoded at this site, the type of gene product (or products) encoded by it or even the nature of the genetic lesion (or lesions) present. Thus, so far, most of the work reported in the literature focuses on the functional and physiological aspects of the lon defect and a detailed genetic characterization is still needed.

Evidence has accumulated in recent years that verifies the regulatory function of the lon locus and its relationship to other complex regulatory systems; in particular, the coordinately regulated group of inducible functions involved in deoxyribonucleic acid (DNA) damage repair known as the SOS functions (67,107,138,184).

The major objectives of this research included mapping of a collection of independently isolated *lon*$^-$ mutations and the phenotypic characterization of each one of these in an isogenic set of Lon$^-$ strains. In addition, characterization of the type of mutation in each strain by mutagen-induced reversion, as well as determination of the degree of complementation and recombination between *lon*$^-$ mutations by construction of merodiploids carrying different *lon*$^-$ alleles were achieved. Attempts were also made to isolate and characterize *lon* deletions.

REVIEW OF THE LITERATURE

A. The SOS Repair System.

The SOS system is composed of a group of inducible functions coded by a number of unlinked cistrons, which are involved in the repair of damaged DNA (67,93,107,126,138,184). The damage can be caused by a variety of sources (See section A). In addition, inhibition of DNA replication by such treatments as incubation with nalidixic acid (91,163), crystal violet (177), or thymine starvation (178) and the presence of temperature-sensitive DNA synthesis mutations [i.e., *lig* (ts) (temperature-sensitive ligase) (31)] lead to the induction of the SOS functions. The complex response includes a number of apparently unrelated phenomena such as increased ability to repair DNA (both excision and post-replication repair) (184), increased mutagenesis (183, 184), inhibition of cell division, filament formation (138,181,184), enhanced induction of λ prophage (181,184,185) appearance of new initiation complexes at the replication origin (82), and the establishment of stable DNA replication (100).

The system that controls the SOS response involves the interaction of two proteins, the LexA repressor and the RecA protease (77,91,107,147, 184). Thus, *recA*$^-$ (recombination-deficient) or *lexA*$^-$ (X-ray sensitive) mutants are reversible, alternating between the induced and non-induced states and allowing for a period of recovery after induction for the repair mechanisms to function, leading to eventual return to normal growth conditions after the damaged DNA has been repaired (107).

Several recent studies have indicated that the signal responsible for triggering the SOS response may be intermediates of DNA metabolism (single stranded DNA or oligonucleotides) and that their target is the RecA protease (107). A general model of the SOS regulatory system is shown in Fig. 1 and a list of relevant genes involved in the response are listed in Table I.

The sequence of events initiated by the inducing signal has been determined experimentally and is known to occur as follows. In the uninduced state, the LexA protein binds at the regulator sites of several unlinked operons involved in the SOS response, including recA and the lexA operon itself (13,166). Upon the generation of the inducing signal (DNA damage), the RecA protease is activated which is then able to cleave and inactivate the LexA repressor, thus alleviating the repressed state of the SOS target genes. These are subsequently transcribed and their messages translated into proteins with the concomitant expression of the secondary SOS functions. Upon completion of DNA repair, with the proteolytic capabilities of the RecA gene product reduced because of the disappearance of the signal (inducing) molecule, the levels of LexA repressor consequently increase and re-establishment of the repressed state ensues (67,107,138,184).

Mutations in the recA gene (zab, lexB) (26,59) (See Table I) abolish the induction of the SOS system without impairment of recombination ability (26,107). In these mutants, the RecA protease is unable to cleave the LexA repressor (67). Induction of SOS functions

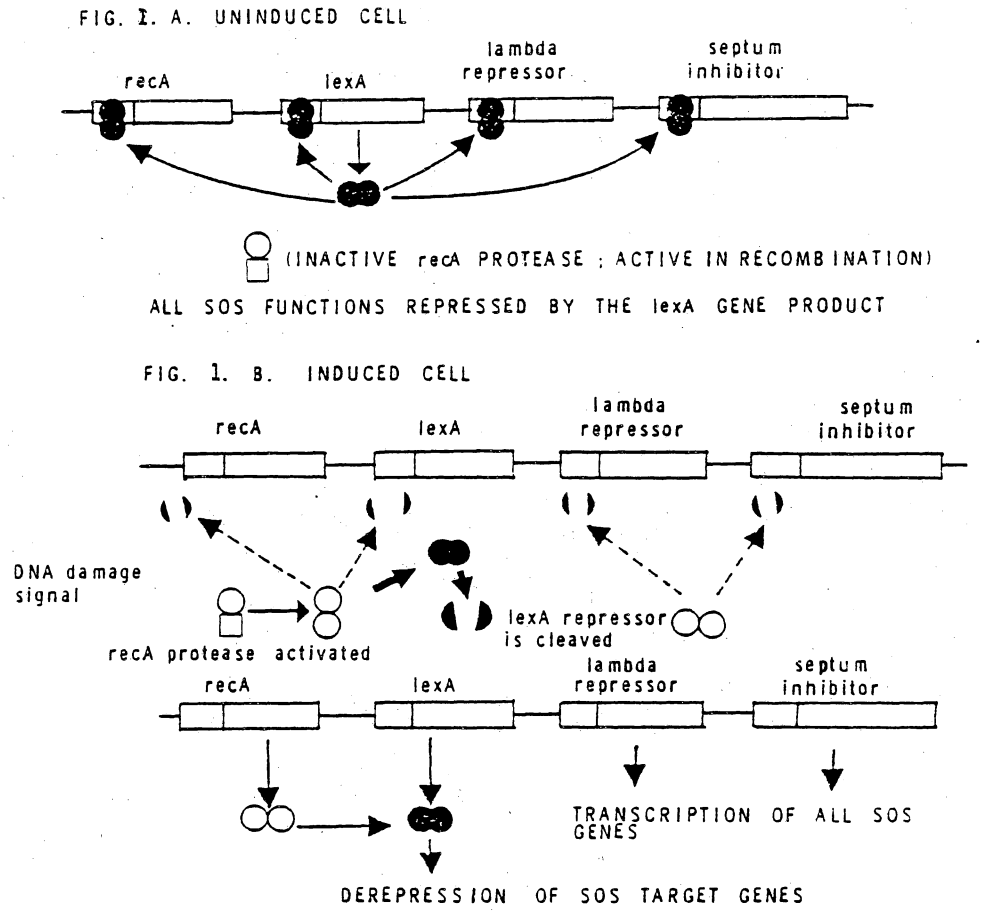

Figure 1. Mechanism of the SOS response.

Table I. Genes Involved in the SOS Response[1]

Gene	Map Location	Function*
recA (tsl,tif, zab,lexB)	58'	General recombination, protease
lexA	91'	SOS repressor
uvrA	92'	Excision repair
uvrB	17'	Excision repair
sfiA (sulA)	22'	Cell division inhibitor (?)
dinA	2'	N.D.
dinB	~8'	N.D.
dinD	80-85'	N.D.
dinF	91'	N.D.

[1]Adapted from reference 107.
ND = not determined.

*Obtained from various sources. See text for details.

can also be abolished by mutations at the lexA locus by which the LexA repressor becomes resistant to degradation by the RecA protease (59,107,184).

The LexA protein controls the damage-induced transcription of at least several genes or operons (See Table I). Kenyon and Walker (96) have demonstrated the existence of a certain class of promoters which are transcriptionally regulated by the SOS system. Studying strains in which the lacZ gene had been fused randomly to promoters using the Mu d (Ap^R, lac) phage, they identified those with expressed levels of β-galactosidase upon treatment with an SOS-inducing agent. Therefore, it was concluded that these promoters had been transcribed under the influence of the SOS system. Among these damage-inducible (din) genes, the uvrA and uvrB genes (UV-repair) of excision repair have been identified (96,148,188). Using another technique, Huisman and D'Ari (81) were able to demonstrate that the synthesis of the sfiA (sulA) (suppressor of filamentation or of lon) gene product increased rapidly during a period of thymine starvation, or after exposure to low fluences of UV irradiation (1erg/sec/mm^2); moreover, this response was directly regulated by the amount of RecA and LexA gene products.

B. The Phenotypes of Lon^- Mutants.

1. UV Sensitivity and Filamentation. Different types of UV sensitive mutants have been described in Escherichia coli K-12 many of which are defective for a particular enzymatic activity involved in one or more pathways of repair of damaged DNA (138,184). Examples of these

are the uvr$^-$ mutants which are involved in the excision repair of non-instructive photoproducts found in the DNA (184). The conditional cell division defective Lon$^-$ mutants are mucoid on minimal medium and form filaments on rich medium when exposed to low doses of UV irradiation or any other radiomimetic treatment (176). The inhibition in cell division observed in Lon$^-$ strains can be suppressed by such treatments as plating the exposed cells on minimal medium, or on rich medium containing 0.1 M pantoyl lactone (63,132,175), or even by just allowing for growth under anaerobic conditions (1). The UV sensitivity of these mutants has been proven not to be due to a loss in the ability to repair UV-induced photoproducts (e.g. thymine dimers) present in the damaged DNA molecule since they are capable of repairing UV irradiated T1 phage DNA and thus differ from the uvr$^-$ class of mutations (49,78,132,175). In fact, Howard-Flanders et al., (78) observed that both uvr$^-$ lon$^-$ and uvr$^-$ lon$^+$ mutants produced the same number of plaques when infected with UV-irradiated T1 DNA, although both were extremely UV sensitive. On the other hand, DNA repair mechanisms of a uvr$^+$ lon$^-$ strain reversed the UV effect (78). Thus, although Lon$^-$ strains had a reduced capacity to tolerate photoproducts in their DNA, this was not the cause for their increased sensitivity to UV (78,182).

Cell division is a precisely controlled process tightly coordinated with chromosome replication in Escherichia coli (74,125). Completion of a round of DNA replication is considered to be a "transition point" that may induce an important sequence of events that enable the cell to

undergo division (12,74,107,125). Results from several studies have demonstrated that filament formation in Lon^- strains is caused by the uncoupling of septum formation from DNA replication. Whenever DNA synthesis is inhibited by UV light, or other treatments, a period of unbalanced growth results in which the ratio of DNA synthesis to total RNA (mRNA) and protein synthesis (DNA/MASS ratio) is lowered (87,91,178). Initially, both wild type and Lon^- strains are equally affected after exposure to agents inhibiting DNA replication (e.g. UV), but Lon^+ cells can return to a normal cycle of cell division after DNA synthesis has been restored, while Lon^- cells are unable to do so (97,104,174,178). Any agent or treatment capable of transiently blocking or delaying DNA synthesis specifically, will induce irreversible filamentation in Lon^- strains (18,63,92,97,178).

Walker and Pardee (132) have observed that treatment of Lon^- strains with the antibiotic nalidixic acid (NA), which inhibits DNA synthesis without interfering with RNA or protein synthesis, caused filamentation in both Lon^+ and Lon^- strains. Upon removal of the drug, whereas Lon^+ cells quickly returned to normal division, the mass increase of Lon^- cells was slower and eventually stopped after two hours. Kantor and Deering (91) also presented evidence supporting the "unbalanced growth" hypothesis. They observed that short incubation periods of Lon^- cells with either NA or hydroxyurea immediately followed by a return to normal growth conditions induced filament formation in *Escherichia coli* B (*fil*$^+$) and *Escherichia coli* K-12 strain AB1899NM

(*lon*$^-$) but not in *Escherichia coli* B / r or *Escherichia coli* K-12 strain AB1157 (*lon*$^+$).

Because the length of the filaments produced was found to be proportional to the amount of protein synthesis that occurred before repair of the damaged DNA had been completed, Witkin (181) hypothesized that the lag period observed in Lon$^-$ strains prior to septation was quantitatively related to the amount of a cell division inhibitor synthesized during the period of derepression.

Nutritional shifts have also been observed to induce filamentation in Lon$^-$ strains and thus, mimic the effect of UV irradiation (104,178). Leighton and Donachie (104) observed that both Lon$^+$ and Lon$^-$ strains re-established the normal DNA/MASS ratio with the same kinetics after starvation; but that the period of time between recomencement of DNA synthesis and cell division was much longer in Lon$^-$ strains, and that the extent of the lag period observed depended on the length of the thymine starvation period. Because of this lag in reinitiation of cell division, filaments were formed (104,178).

Though each independently isolated Lon$^-$ mutant exhibited a characteristic degree of UV sensitivity and filamentation (78), and quantitative differences in Lon phenotypes were apparent even between the same allele in different genetic backgrounds (70), the principal effect of the *lon*$^-$ mutation was to influence re-establishment of cell division in cells in which the DNA metabolism had been blocked (130).

2. The Mucoid Phenotype. In addition to the synthesis of a number of polysaccharides such as the techoic acids and lipopolysaccharides (LPS), which are structural components in bacterial cell walls; a large number of cells are able to synthesize polysaccharides found outside the cell wall and which are excreted into the surrounding environment to form a capsule around the cell (164). Exopolysaccharides have been related to the development of bacteriophage resistance and are also a valuable epidemiological tool in the immunochemical identification of bacterial strains (164,168). Within many of the serotypes found in a large number of Escherichia coli strains and most of Salmonella related species, a specific heteropolysaccharide known as colanic acid is excreted in excess when culture conditions are suitable. Colanic acid is composed of 33% galactose, 16% glucose, 18% glucuronic acid and 33% of the methylpentose, L-fucose (90,164). The hexasaccharide unit contains a pyruvate molecule attached to a terminal galactose residue on a trisaccharide branch found at the central fucose residue of a linear trisaccharide 3-glc-1$\xleftarrow{\beta}$4-Fuc-1$\longrightarrow$4-Fuc-1 and an acetyl group is sometimes found on the first fucose residue (164). Markovitz (113) isolated mucoid mutants of Escherichia coli K-12, which upon further examination were found to be conditionally lethal because of their UV sensitivity. In transductional crosses the mutation (capR) was found to be closely linked to proC$^+$ (min 9) mapping at the same position previously assigned to the lon mutation on the Escherichia coli K-12 circular chromosome map (min 10).

Of eleven enzymes known to be involved in the capsular polysaccharide biosynthetic pathway, at least five have been found to be derepressed in CapR$^-$ (Lon$^-$) strains: phosphomannose isomerase (PMI) (*man*; min 36), uridine-5'-diphospho (UDP)-galactose-4-epimermase (*gal E*; min 17), guanosine diphosphate (GDP)-mannose pyrophosphorylase (*galU*; min 27), GDP-L-fucose synthetase and UDP-glucose pyrophosphorylase. At least four different operons are involved in the production of these enzymes (105,106,113,114,130,137,175). Furthermore, Mackie and Wilson (109) demonstrated that a mutation at the *capR* (*lon*) locus caused a two to four-fold increase in the mRNA levels of the galactose (*gal*) operon with the corresponding increase in enzyme levels. Thus, at least 10 enzymes involved in capsular polysaccharide synthesis seemed to be influenced by the *capR* (*lon*) locus.

Because different *capR*$^-$ mutations confer varying degrees of expression of the mucoidy and UV sensitivity phenotypes (20), it has been suggested that the higher UV resistance exhibited by extremely mucoid Lon$^-$(CapR$^-$) mutants was due to the amount of protection conferred to them from the polysaccharide alone (43).

The relationship between the control of synthesis of excess capsular polysaccharide and the other phenotypes associated with the *lon* defect (UV sensitivity in particular) has been the objective of some investigations in recent years. Markovitz and Rosenbaum (115) have observed that at least two distinct classes of *capR* mutants could be defined based on their pattern of dominance. Thus, in partial diploids

whereas the capR6 mutation was recessive to capR$^+$ regardless of its location, the capR9 mutation was dominant to capR$^+$ when located on the episome, but recessive if on the chromosome (episomal dominance); although in both combinations, the degree of UV sensitivity remained the same. Gayda et al. (62) have further isolated second site revertants by plating Lon$^-$ cells on media containing nitrofurantoin (NF). The revertants retained their mucoid phenotype but had acquired resistance to UV light. Conversely, non-mucoid, UV sensitive revertants have also been isolated (70). Mutations were induced in the gal operon that suppressed mucoidy by blocking the capsular polysaccharide biosynthetic pathway without affecting other Lon$^-$ associated phenotypes. These gal$^-$ lon$^-$ double mutants became mucoid when an F' gal$^+$ was introduced (70). Therefore, the separation of mucoidy from other Lon phenotypes seemed to indicate that overproduction of capsular polysaccharide did not play a major part in the expression of other lon defects.

Hua and Markovitz (80) have suggested that on the basis of the properties of regulation deficient mutants of the gal operon, the capR (lon) gene product is a repressor whose function is independent of the specific repression system for the gal operon.

3. Decreased Ability to Form λ and P1 Lysogens. Following infection of a non-lysogenic Escherichia coli K-12 cell with wild type λ phage, the virus can either enter its lytic cycle which involves the sequential expression of viral genes that will eventually cause lysis of the host cell releasing new viral particles, or it can lysogenize the

host. In the latter state, the phage chromosome stably inserts into a specific site on the Escherichia coli chromosome with the accompanying repression of viral vital functions (22,66,73). The decision or commitment to either lysis or lysogeny is mediated by a system of control circuits involving complex interactions among positive and negative control mechanisms (167). A linear map of the λ chromosome indicating the position of relevant genes and their action as well as a detailed genetic map of the λ cI region are shown in figure 2.

In brief, regulation of gene expression of λ during infection occurs as follows. The λ chromosome is divided into two operons each having its own promoter-operator region, which are transcribed in opposite directions (O_RP_R, O_LP_L). Transcription of early genes from the O_LP_L promoter gives the N gene product while the rightward operon (O_RP_R) gives the cro gene product. Transcription stops immediately after these two genes but the antiterminator action of the N gene product allows synthesis to continue into the cII, O, P and Q genes on the rightward operon and to cIII on the leftward operon (45,66,176). The N and Q gene products are positive regulators which permit transcription of other late phage genes. (See figure 1.) The cII and cIII gene products stimulate the synthesis of cI gene product, the λ repressor protein, from the P_{RE} promoter (promoter for repressor establishment) which is able to recognize the two operator sites O_L and O_R. Nevertheless, the cro gene product can inhibit the synthesis of cII and cIII proteins thus preventing the expression of cI (136). In this way the cro gene product

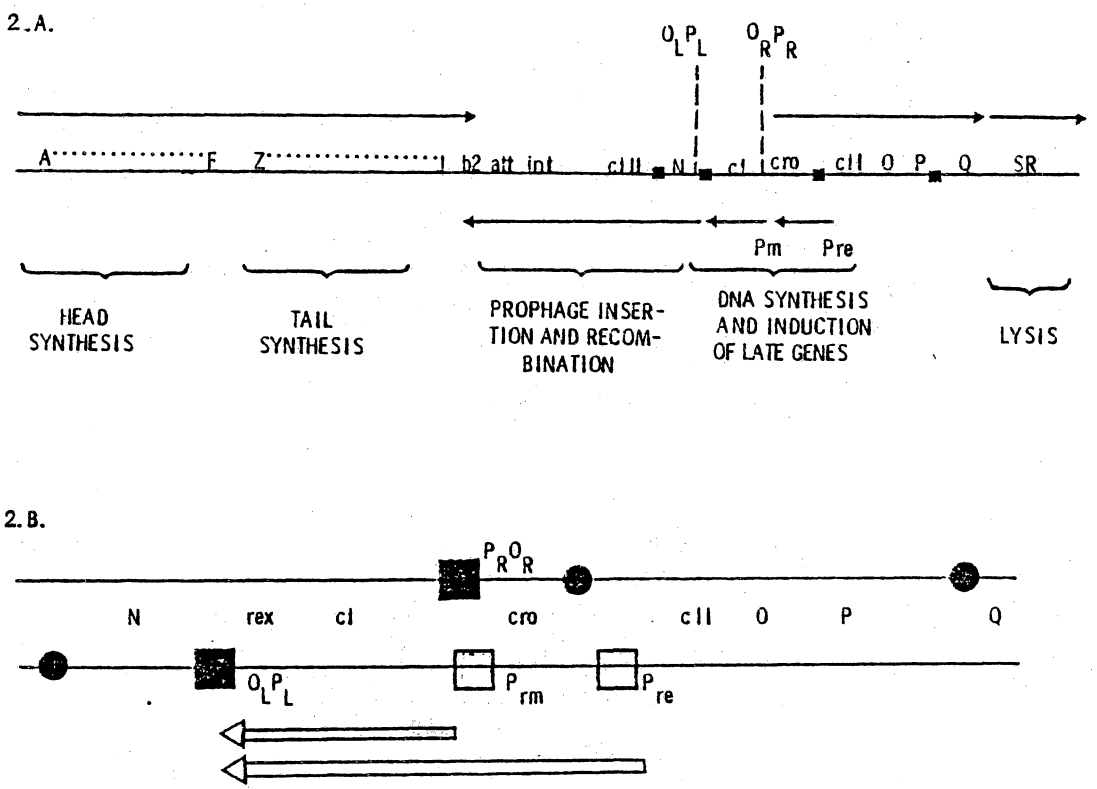

Figure 2. Map of the λ chromosome. (A) Simplified map of the entire λ chromosome. Arrows indicate direction of transcription. Black boxes indicate transcription termination signals. Dotted lines represent genes found between the two genes shown at the ends of each region. (B) Map of the early region of the λ chromosome showing genes involved in the control circuits that regulate lysis and lysogeny. Boxes indicate promoters. Circles represent transcription termination sites. (11,22,45,47)

becomes the most important regulatory protein involved in the establishment of lysogeny or the induction of the lytic pathway since it blocks leftward transcription and synthesis of the N antiterminator preventing transcription of cIII thus reducing cI repressor synthesis (47). Furthermore, the cro protein can also interact with the O_L and O_R sites, and thus competes with the cI repressor displacing it from the operator sites to alleviate repression and give then the lytic cycle (11,160). Therefore, if insufficient cro protein is produced, then λ N gene product stimulated synthesis of cII and cIII will increase the levels of λ cI repressor resulting in the establishment of lysogeny (22,47,136).

It is now known that the induction of λ prophage that follows UV irradiation of a λ lysogen occurs as a result of the proteolytic cleavage of the λ cI repressor by the RecA protease (67,107,139). As implicated above, whereas the cI, cII and cIII genes are involved in the establishment of repression, the cro and λ cI gene products alone seem to be involved in the maintenance of the lysogenic state. Wild type λ phage forms turbid plaques on Lon^+ strains but clear plaques on Lon^- cells (43,68,130,169,179). This has been interpreted as being the result of a reduced capacity of the phage to express certain repressor genes involved in the establishment of lysogeny whenever it is introduced into the lon^- genetic background (169,179). In fact, this inability to form lysogens is evidenced by the greater spontaneous liberation of free λ phage from lon^- (λ) lysogens as compared to lon^+ (λ) lysogens (179).

Truitt et al. (169) have reported a decrease of λ cI repressor levels in Lon$^-$ strains to about 50% of the levels observed in Lon$^+$ strains after infection with λ. Previously, Walker et al. (179) had demonstrated that infection of Lon$^-$ strains by λ cII$^-$, cIII$^-$ and cro$^-$ mutants, which are defective in the establishment of lysogeny, had lower λ lysogenization frequencies in Lon$^-$ hosts. It was later demonstrated by Truitt et al. (169) that the lon$^-$ effect on repressor activity was not due to an altered interaction of the lon gene product and the cro repressor since upon infection of Lon$^+$ and Lon$^-$ cells with the temperature-sensitive λ cI857cro27 mutant phage, which synthesizes repressor constitutively, differences in repressor levels persisted. In addition, these investigators observed that since lon$^-$ and lon$^+$ λ$^+$-monolysogens could be isolated in which repressor levels were the same, the lon$^-$ mutation was directly involved in the establishment of repression but not in the maintenance of the lysogenic state. Takano (165) and Falkinham (52) further proposed that in a similar way both the P1 lysogeny defect and the deficiency in inheritance of F plasmids, respectively, were due to the impaired ability of establishing these elements in Lon$^-$ cells, because once inherited in Lon$^-$ mutants they are retained by the progeny. Takano (165) has proposed that the presence of the lon$^+$ gene is indispensable for the establishment of lysogeny and that the commitment to lysogeny may be blocked in Lon$^-$ strains due to inhibition of either transcription of the λ N cistron or the failure of the P1 immunity repressor to operate.

The evidence gathered suggested that a common control mechanism regulated both induction of λ prophage and cell division inhibition after UV irradiation (60,63,67,68,75,129,181). These two phenomena are also known to be part of the group of inducible SOS functions that are turned on whenever DNA is damaged by either ionizing radiation, DNA-alkylating or cross-linking agents or other radiomimetic treatments. Mutants at the recA gene have been isolated that are temperature sensitive and able to trigger the SOS response at 41C in the absence of DNA damage (See below). This mutation is called tif (temperature induced filamentation) and maps at min 58 within the recA gene Escherichia coli K-12 chromosome map (121). In tif⁻ lon⁻ double mutants though filamentation is enhanced neither prophage induction nor repair of UV irradiated phage DNA are induced (63). Thus, George et al. (63) have suggested that the latter two phenomena may not be directly affected by the lon⁻ mutation and that these two functions are under the control of different mechanisms exhibiting different senstivities to the same DNA damage signal.

4. Degradation of Proteins. In bacteria, adaptive and physiological changes in the cell are mediated by enzymes and regulatory proteins whose levels are controlled by specific proteases (130). The processes of phage and cell morphogenesis as well as secretion of proteins through the cytoplasmic membrane are controlled in similar fashion (130). In Escherichia coli most polypeptides are stable and exhibit a low turnover rate (65). In non-growing bacteria, about 5-12%

of the total cell protein is degraded per hour; and gross structural alterations of normal proteins lead to rapid *in vitro* degradation (65,193). A system able to distinguish between normal and defective polypeptides is known to exist in *Escherichia coli* K-12 (17,28,123,128, 154,193). Nonfunctional proteins such as nonsense polypeptide fragments (154) and some classes of missense proteins (70) as well as proteins containing structural analogs (65) are recognized by this system and rapidly degraded.

Bukhari and Zipser (17) isolated a class of mutants (*degT*) with increased ability to stabilize nonsense polypeptide fragments. Apte *et al*. (4a) have isolated and characterized a second class of Deg^- mutant (*degR*) which were unable to degrade polypeptide fragments that resulted from reinitiation of transcription past nonsense mutations. Both mutations were found to be linked to *proC* and exhibited the same range of Lon phenotypes (70,154). Further, Lon^- and $CapR^-$ mutants were shown to display the Deg^- phenotype (70).

A proteolytic activity of the lon^+ locus has been identified (27,28,130). Burgess *et al*. (19) isolated temperature-sensitive mutants in which the sigma subunit of the ribonucleic acid (RNA) polymerase had been altered (*rpoD800*). In Lon^+ cells, after a shift to the nonpermissive temperature, RNA and protein synthesis were diminished because the inactive, mutant sigma factor was completely degraded. By contrast, in a lon^- background, the defective sigma factor was stabilized. Gottesman and Zipser (70) screened λ and T5 temperature-

sensitive missense mutants for growth on Lon$^+$ and Lon$^-$ strains at the nonpermissive temperature. These investigators observed improved efficiency of plating on Lon$^-$ strains. According to their interpretation, the missense mutations had been suppressed because the slow degradation rate in Lon$^-$ cells allowed for their expression. This could then explain why, in some cases, apparent missense suppression occurs. As happens with the λ *cro*$^-$ mutation, suppression mediated by *lon* may not be due to a direct regulation on the specific gene but to the stablization of a defective gene product which allows for its residual activity to be expressed (70).

It may well be that the *lon*$^+$ gene product performs a regulatory function responsible for the degradation of a hypothetical division-inhibiting protein, in the absence of which, Lon$^-$ mutants are sensitized to SOS inducing treatments (84). Thus, the exaggerated response to DNA damage and its consequent inhibition of replication in *Lon*$^-$ backgrounds could be due to stabilization of a repair-associated division inhibitor (RADI) (74).

The part that the Deg system plays in the control of the establishment of λ lysogeny has been assessed recently by Gottesman and co-workers (68). At least three classes of early λ proteins were identified on the basis of their metabolic fate in a *lon*$^-$ background. Whereas in Lon$^-$ mutants the chemical half-life of the normal λ N protein was increased five-fold, that of the λ cII gene product decreased by 50%. A third class of proteins, represented by the λ O gene product,

remained unaffected. Thus, in addition to nonsense, missense and structurally altered polypeptides, the *lon*$^+$ gene product also exerted a specific effect on the degradation of wild type proteins (68). The stabilization of the λ N protein and decrease in stability of the cII gene product in the cytoplasm of a Lon$^-$ cell could eventually result in lysis, an observation noted by Takano (165) and Gayda and Markovitz (60).

5. *Mutations Epistatic to lon*. Second site mutations which are able to restore the wild type (or near wild type) character to a mutant phenotype are known as suppressor mutations. Ochre and amber suppressor mutations are capable of reverting nonsense but not missense mutations (14,62,73,160). Both amber and ochre mutations are able to generate polarity effects which are relieved by nonsense suppressors. To dissect the genetic nature and functional character of the *lon* defect, Markovitz and Baker (114) studied the effect of several nonsense suppressors on the expression of the *lon* gene(s) and observed that different suppressor mutations were able to revert either the UV sensitivity or the mucoid phenotype to a different extent. Whereas *supF* was most effective in reverting the mucoid phenotype, *supC* was found to restore the UV resistant phenotype with more efficiency. In their experiments, the presence of *rho*, a nonsense polarity suppressor, had no effect on either UV sensitivity or mucoidy in a *capR9* strain. They concluded that the product of the *capR9* (*lon*) locus was a protein because the effect of an ochre suppressor mutation occurs at the level of mRNA translation. The

fact that mucoidy and UV-sensitivity were not coordinately suppressed suggested (though not to the authors) that either two genes made up the lon locus or that different portions (domains) of the molecule with different activities (e.g. UV-repair and regulation of capsular polysaccharide synthesis) are affected differently by nonsense suppressors.

Genetic studies of the lon locus have been facilitated by the isolation of second site suppressors able to restore one phenotype caused by the lon defect, but not others (88, 89, 137). Radke and Siegel (137) described some Lon$^-$ strains in which a his-linked mutation (min 44) successfully suppressed mucoidy without affecting UV sensitivity. This mutation, called non (min 45), prevented strains to develop resistance to T7 phage and inhibited capsule formation. non capR9 double mutants accumulated large amounts of nucleotide precursors to capsular polysaccharide (61). Loss of the ability to synthesize a capsule somewhat increased the degree of radiation sensitivity of the same mutation (61).

Mutations have been isolated and characterized (88,89) which are able to restore UV resistance, eliminate filament formation and alleviate the λ lysogenization defect. These revertants were isolated as methyl methane sulfonate (MMS) resistant (MMS^R) (114) or nitrofurantoin (NF)-resistant (NF^R) clones (62). These mutants, called sul, have been found to map at two distinct loci in the E. coli chromosome; sulA is co-tranducible with pyrD (min 22) and sulB with leu

(min 2) (69,88,89). The sul mutations studied suppressed the Lon$^-$ phenotype to different extents (69,87,114). In all cases the lon$^-$ sul$^-$ double mutants suppressed UV sensitivity, while increased capsular polysaccharide production and the protein degradation defect were not abolished (62,69).

Each sul mutation exhibits a distinct dominance pattern. It has been found that sulA is recessive to sulA$^+$ whereas sulB was found to be dominant to both lon$^-$ and sulA (69). (See below). George et al. (63) isolated suppressed temperature-resistant revertants of a tif lon$^-$ double mutant able to restore division at the nonpermissive temperature, that exhibited phenotypes identical to those of sulA (sfiA) and sulB (sfiB) suppressed Lon$^-$ strains, and which mapped in the chromosome as those mutations (63,69,82,83).

The frequency of mutations at the sulB locus was found to be much lower than that observed for mutations at the sulA locus (69) and whereas sulA insertions have been isolated, this has not been the case with sulB (82). Because of this evidence and the fact that sulB strains tend to filament spontaneously in the absence of any DNA damaging agent, it has been suggested that, although both sulA and sulB are clearly involved in the filamentation response, their respective functional roles in this process are different (62,69,74,81,82). George et al. (63) and Gottesman et al. (69) have indicated that the possible role of the sulA$^+$ gene product, as the inhibitor of septum formation, may result from its reduced degradation rate in Lon$^-$ cells and thus is equivalent

to the proposed RADI; since stabilization of the *sulA*$^+$ gene product in a *lon*$^-$ background could lead to irreversible lethal filamentation (69,82). On the other hand, *sulB* mutations may define a specific site which is a structural component of the cell division apparatus and that may be the target site of the *sulA* gene product (RADI) resulting in a cell division block (63,69).

Gottesman *et al*. (69) proposed that at least two filamentation systems exist in *E. coli* K-12. The first, temporary or transient filamentation, is exhibited by both Lon$^-$ and Lon$^+$ cells regardless of the state of *sulA* and *sulB*. The second, *sul*-dependent (*sfi*-dependent; 78) filamentation, is observed only in *lon*$^-$ *sul*$^+$ (*sfi*$^+$) cells and leads to irreversible cell division inhibition. This second filamentation system has been proposed as the one responsible for the enhanced UV sensitivity of Lon$^-$ cells (82).

Because some *sulA* (*sfiA*) and *sulB* (*sfiB*) mutations have been found that are either dominant or recessive (63,69,107), it has been suggested that these two loci form part of an even more complex regulatory system. Furthermore, *sul* (*sfi*) mutations are also known to block SOS-induced filamentation and; in particular, *sulA* appears to be under the control of the *lexA* gene, whose gene product, the LexA repressor, interacts with the RecA protease to control the SOS functions. Direct evidence for this comes from Huisman and D'Ari (81) who have been able to demonstrate that the *sulA* operon is UV-inducible (a *din* function) and thus forms part of the SOS response. This induction was the same in both *lon*$^-$ and

lon$^+$ *sfiA*::*lacZ* fusion strains and suggested that the *lon* gene product is not a specific regulator of *sulA* transcription.

The relationship between the *lon* and SOS systems is well documented (63,67,107,184) (See section C). It has been shown that mutations at the *exr* (*lexA*) and *recA* loci reduce the lethal effect of filament-inducing agents or treatments which cause direct damage to DNA (36,82,87). George *et al*. (63) presented evidence that Lon depends on the RecA gene for its expression since *recA*$^-$ *lon*$^-$ double mutants continued to divide almost normally after treatment with UV. In *sul*$^-$ *lon*$^-$ *recA*$^-$ or *sul*$^-$ *lon*$^-$ *lexA*$^-$ strains, the resistance to UV irradiation otherwise conferred by the *sul*$^-$ mutation, is masked by the radiation sensitivity caused by *recA*$^-$ and *lexA*$^-$ mutations. In contrast to the *sul* mutations which do not suppress lysis, a *recA*$^-$ or *lexA*$^-$ mutation is able to block cell lysis (41,87).

Proteases have been implicated in the control of the expression and induction of the SOS functions. The mechanisms involved are thought to be similar to the proteolytic cleavage of λ repressor by the RecA protease (139). Thus, the *lon* gene product, by virtue of its Deg$^-$ phenotype, could be involved in the modulation of the SOS response since the increased stability of the proteins synthesized during the very brief period of SOS induction could be the result of the altered rate of polypeptide degradation in Lon$^-$ mutants (70,184).

C. Interaction of the SOS and Lon Systems.

The kinetics of RecA protease induction were found to be similar in Lon^+ and Lon^- strains (36); however, the kinetics and rifampicin sensitivity of UV-induced septation inhibition were different from those observed for RecA protease synthesis (84). This lack of agreement suggested basic differences in the molecular mechanisms controlling these two responses. Further indications of possible divergent patterns of expression and control in the SOS and Lon systems came from experiments involving temperature-sensitive recA mutants. Huismann et al. (84) observed that filamentation could be induced in tsl and tif (temperature induced filamentation) mutants of recA in the absence of RecA protesae synthesis. At 41C, tif cells formed filaments, showed increased DNA repair and mutagenesis, and enhanced induction in λ lysogens, all reverted by recA, zab and lex mutations (25,26,63). Because this pattern is different from that seen in Lon^- strains, different targets for their action must be proposed (63). Huisman et al. (78,82) have proposed a more specific involvment of the lon locus in the process of cell division with no direct influence on other manifestations of the SOS response, based on the observation that lethal irreversible filamentation can be induced in Lon^- strains at UV fluences too low to induce the SOS response.

Because of the Deg^- phenotype, many investigators have speculated that the extreme radiation sensitivity of Lon^- cells to SOS-induced filamentation could be due to the absence of the Lon^+ protease normally

involved in the elimination of the sulA gene product, whose synthesis is enhanced as an SOS function. The stabilization of the sulA gene product (the RADI) would then prolong cell division inhibition whenever Lon$^-$ cells become exposed to SOS inducing treatments (63,81,82,107,184).

D. Isolation and Characterization of the Lon Gene Product.

It has recently been demonstrated that one product of the lon locus is the ATP-dependent protease, protease La (27,28). Zehnbauer and Markovitz (190) have cloned a DNA fragment capable of suppressing Lon$^-$ phenotypes from the plasmid which carries lon. Two polypeptides were coded by that 8.2 megadalton (Md) fragment: a 94 kilodalton (94 K) polypeptide and a 67 K polypeptide. The 94 K polypeptide specified by the CapR9 mutant was different from wild type (lon$^+$). Zehnbauer et al. (189) provided further evidence that the 94 K polypeptide was indeed the lon (capR) gene product and they further showed that the protein exhibited single-stranded and double-stranded DNA binding ability. Schoemaker and Markovitz (149) presented evidence which showed that plasmids having terminal deletions of the fragment coding for the 94 K polypeptide were nonfunctional with respect to capR$^+$. Therefore, it was suggested that the 94 K polypeptide was not only essential for the expression of the capR (lon) function, but that it was also sufficient. The 67 K polypeptide was considered not to be implicated in the expression of the capR$^+$ (lon$^+$) gene. However, at least one of the plasmids carrying a deleted 94 K DNA coding fragment showed some suppression of mucoidy when this plasmid was used to transform capR$^-$ recipients to the wild type phenotype (149).

Chung and Goldberg (28) purified the Lon protein from strains RGC121 (*lon*$^+$), RGC121/pJMC40 which carries the *lon*$^+$ allele in both the chromosome and the plasmid pSC101, and RG123 (isogenic with RG121) and reported that it is a tetramer composed of four identical units; with a molecular weight of 450,000 daltons. It was shown to be identical to the protease La by the following criteria. The Lon$^+$ protease was found to have an ATP-dependent proteolytic activity with the cleavage of ATP and the presence of Mg^{++} cations being essential for proteolysis. This last requirement differentiates the *lon* gene product (protease La) from other known proteases (28). Protease La was found to be present in Lon$^-$ cells in reduced amounts and, although the degradation of abnormal proteins proceeded at a slower rate, the process still showed a requirement for energy. Failure to find mutants in which the Lon protease activity was truly absent suggested that this gene could be essential for cell survival (28).

Charette *et al*. (27) further observed that the gene product purified from a *capR9* mutant that exhibited the Deg$^-$ phenotype, was lacking the ATPase-dependent protease and ATP-hydrolysis activities although the DNA binding activity remained; thus, indicating a separation of functions in the *lon* gene product similar to that observed among mutants of the RecA protease.

It has been suggested that higher levels of ATP could result in the increased cleavage of a radiation-associated division inhibitor (RADI), or increased activity of proteins needed for division (inactivation of

repressors or direct proteolytic activation) (27). Given the dependence of the Lon protease on ATP hydrolysis, the interaction between pools of nucleotides and nucleic acids may regulate the *lon* (*capR*) gene product *in vivo* (27).

E. The Mechanism of Lon Action: A Hypothetical Model.

From the foregoing discussion it is clear that the one important feature of the *lon* gene product is its proteolytic activity (27,28,68,70,184,189). The role of the Lon protease as a modulator of the expression of other genes is evident. The discovery by Zehnbauer *et al*. (189) that the *lon* gene product has DNA binding ability could help explain its mode of interaction with the *gal* operon and the several related cistrons involved in capsular polysaccharide synthesis in *Lon*$^-$ mutants.

A hypothetical model synthesizing several already proposed models which try to explain the diversity of phenotypes associated with the *lon* defect is shown in Fig. 3. This model accounts for the possible dual role of the Lon protease based on the evidence presented so far. In the interpretation of the model, the following assumptions have been made for the sake of simplification: (1) that only one mutational lesion causes the *lon* defect and (2) that only one gene is affected at the *lon* locus. As noted below, these assumptions do not exclude other interpretations. The sequence of events proposed in a *lon*$^+$ *sul*$^+$ *recA*$^+$ cell is shown in Fig. 3A. After the appearance of an inducing signal (i.e., UV photo-products), the RecA protease would be activated and the LexA repressor cleaved. In lysogens, the repressor would be also

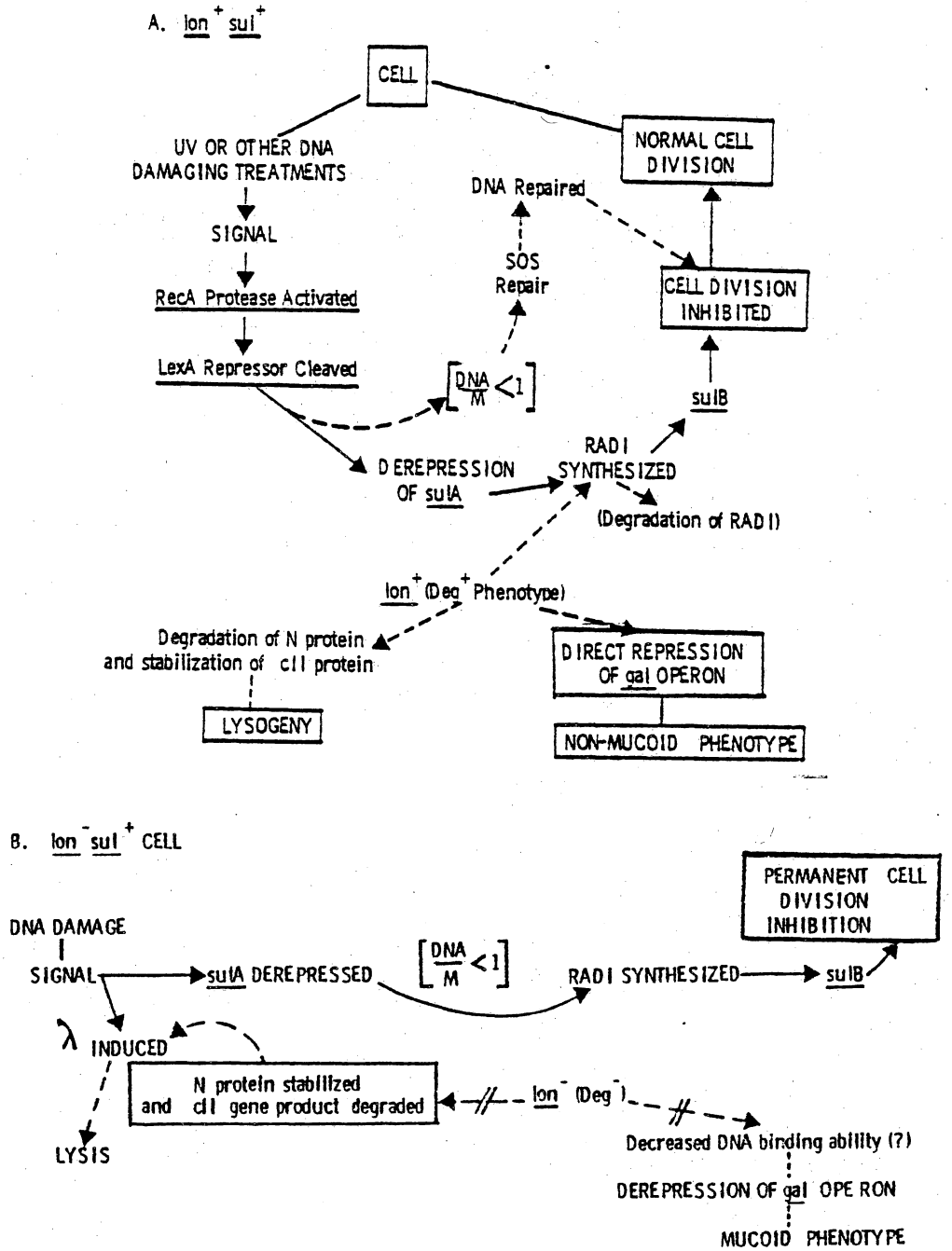

Figure 3. Model for the mechanism of Lon action. Adapted from several sources (62,63,69,81,82,83,84,87,107,184).

cleaved by the RecA protease resulting in cell lysis. Induction of the entire group of SOS functions, of which sulA (sfiA) is part, would then follow; consequently, the levels of the sul gene product, the RADI, would be increased. The continued increase in RNA and protein, during a period of DNA synthesis inhibition would then account for the decrease of the DNA/MASS ratio. The RADI would then act directly at the structural site where septum formation occurs; the site coded by $sulB^+$. This would result in inhibition of cell division and the consequent production of filaments. After completion of DNA repair, and the re-establishment of the normal pre-irradiation DNA/MASS ratio, the Deg phenotype could be expressed; thus, the RADI would be degraded and the inhibition of septation (slower in Lon^-) abolished.

In the same fashion, degradation of the λ N antiterminator protein and simultaneous stabilization of the λ cII gene product, would result in the return to the lysogenic state.

To accomodate the suppression of mucoidy, the model assumes that, by virtue of its DNA binding capacity, the Lon gene product would be able to directly repress the gal operon by binding at its operator-regulator site and in similar fashion, to the other operons involved in capsular polysaccharide biosynthesis, resulting in a nonmucoid phenotype.

Figure 3B presents the same sequence of events in a $lon^- sul^+ recA^+$ cell. Again, derepression of sfiA (sulA) would lead to increased levels of the RADI which would block division at the $sulB^+$ site on the cell

wall. Simultaneously, λ prophage induction would occur in lysogens leading to lysis. The lack of Lon protease would then help stabilize the RADI resulting in a longer inhibition of septation. Concomitantly, the Deg^- phenotype would lead to long half life of the λ N antiterminator with the resulting decrease in λ cII repressor that could result in lysis. It is possible that stabilization of proteins contributes to the extended period of time observed for the return to the normal DNA/MASS ratio observed in Lon^- strains; therefore, even after DNA damage is repaired, Lon^- cells would be unable to recover from the septation inhibition block. The same defect that reduces the proteolytic activity of the lon gene product could also affect its DNA binding ability in a way that could result in decreased affinity for the operator-regulator region of the operons controlling polysaccharide synthesis, including the gal operon; thus, causing their derepression and consequently producing the mucoid phenotype.

The model accounts for several features of the Lon phenotype described in several models already proposed (62,63,69,81,82,83,84, 87,107,184). It also shows the possible interaction of the Lon and SOS systems. Hence, it can be seen that in $lon^- sul^+$ mutants, lethal filamentation would be the result even if DNA repair were to occur normally. In similar fashion, the defect in lysogenization of λ could be explained. It can also be seen from this model that a mutation in recA and lexA or even in both, would revert the filamentation defect since the RADI would not be synthesized because of the repressed state

of the sulA operon. Similarly, mutations at sulB, even in the absence of the Lon protease and sulA gene products, could result in the permanent restoration of division.

The uncoupling of the filamentation response from the other aspects of the SOS response that is seen in Lon^- mutations (84) could be explained by this model. In similar fashion, the apparent capacity of Lon^- strains to suppress leaky missense mutations could also be explained by the model since in Lon^+ strains, a rapid turnover of the missense polypeptide would account for the mutant phenotype, whereas in Lon^- strains the stabilization of the abnormal protein would allow for the expression of a somewhat defective but functional phenotype (70).

This model could also be interpreted in the same way if there were two genes at the lon locus, each with a distinct lesion; or one gene with two distinct mutations, each affecting the structure of the same gene product in a different way, resulting in different functions. This has to be taken into account because, in spite of the fact that the lon gene has been cloned and its product characterized, the genetic structure of the lon locus has not been clearly defined. In addition, the fact that the Lon protease is a tetramer and the suggestion that interactions of defective and normal monomeric units (62) could influence such features of lon gene expression as episomal dominance, make the involvement of more than one lesion at this locus, an attractive possibility.

F. The Possible Genetic Structure of the lon Locus.

In spite of the cloning of the lon gene and the isolation and characterization of its product as a 450,000 dalton tetramer of identical subunits (27,28,189), some puzzling results, such as the presence of a second 67 K polypeptide and the inability of some deleted fragments of the 94 K coding DNA to confer the entire range of wild type phenotypes (capR$^+$) to capR$^-$ recipients during transformation experiments, suggest the existence of multiple lesions at one locus from which diverse phenotypes would result. Alternatively, the presence of at least two distinct genes or of two tandem copies of one gene, each one of which would express different phenotypes due to different, independent mutations is also possible. In each of these cases, the hypothesis of a separation of functions in one gene product could be accounted for, since mutations at different points along the locus could be associated with the loss of specific functions.

The separation, in the Deg$^-$Lon$^-$ nonsense mutant CapR9, of the protease and ATPase activities from the DNA binding capacity (28) could be explained either by different lesions in different genes, multiple lesions in one gene, or a single mutation affecting different domains to varying extents.

The first evidence strongly suggestive of the existence of two genes at the lon locus came from the work of Donch and Greenberg (42). Among a collection of UV sensitive mutants obtained by direct selection of mucoid clones on minimal glucose medium at 37C, UV sensitive mucoid

($UV^{S}M$) and UV resistant mucoid ($UV^{R}M$) colonies were found. The degree of resistance was characteristic of each clone and thus suggested the existence of at least two phenotypic classes on the basis of UV sensitivity. Transductional analysis showed that the more UV resistant clones had a higher co-transduction frequency with *proC*$^{+}$ (24 - 29%) whereas the UV sensitive class showed less linkage with *proC*$^{+}$ (12 - 17%); thus, suggesting the presence of two genetic "blocks" which controlled UV sensitivity (42). Although recombination analyses from interclass (e.g. $UV^{S}M$ x $UV^{R}M$) crosses produced Lon^{+} recombinants, intraclass crosses (e.g. $UV^{R}M$ x $UV^{R}M$) failed to do so. Complementation analyses between classes also gave no positive results. This apparent contradiction was interpreted by Donch and Greenberg as an indication of two mutations present in one cistron (42).

When the degree of UV sensitivity is taken into consideration, independently isolated Lon^{-} strains fall into either one of the two phenotypic classes: class A is characterized by low UV sensitivity while class B includes highly UV sensitive strains. Thus, strains AB1899 (*lon-1*) (78), AX14 (*lon-8*) (175) and MC104 (*capR6*) (114) belong to class A, whereas strain PAM550 (*lon-550*) (42) and the strains carrying the alleles *lon-205* and *lon240* (165) as well as strain MC102 (*capR9*) (113) can be included in class B. Markovitz and Baker (114) reported that *capR6* which was found to be more closely linked to *proC*$^{+}$, conferred more UV resistance than either *capR9* or *capR66* which were found to be weakly linked to *proC*$^{+}$. Furthermore, *capR9* and *capR6* were able to recombine

and produce Lon$^+$ recombinants. Bush and Markovitz (20) suggested that a gradient of UV sensitivity in these strains could be due to a gradient of lessions in the genetic map since in several Lon$^-$ mucoid strains the differences in UV sensitivities observed seemed to correspond to the linear order of the genetic lesions at the lon locus.

Zehnbauer and Markovitz (190) observed that capR$^+$/pBZ201M9 (a capR9-carrying plasmid) transformants were UV sensitive although mucoid and NF resistant, in contrast to the previous finding that capR$^+$/F'capR9 partial diploids were mucoid but UV resistant (114). These investigators further observed that spontaneous mucoid segregants could be obtained that were able to yield capR recessive plasmids which were not able to transform capR9 or capR$^+$ recipients to the wild type phenotype. Nonmucoid clones were also segregated which were able to confer sensitivity to nitrofurantoin and mucoidy upon transformation to wild type strains.

Because of the phenomenon of episomal dominance observed with the capR9 mutation, the difference in degree of expression of each mutation and the diverse patterns of effectiveness of nonsense suppressors in restoring the nonmucoid or the UV resistant phenotypes, Markovitz and Baker (114) had proposed that mucoidy and UV sensitivity could be controlled by either one gene or two genes in the same operon. These investigators further suggested that the negative complementation observed when capR9 was episomal, could have been due to the production of defective protein monomers that upon combining with the normal

monomers coded for by the *lon*$^+$ (*capR*$^+$) on the chromosome, produced an inactive oligomer (114).

From the evidence reviewed here, although the details of regulation and control of the expression of the *lon* locus seem to have been explained; still, a more detailed correlation between genetic structure and function is needed. Thus, in brief, a more complete genetic analysis involving complementation studies could improve our understanding of the *lon* locus' genetic structure and its relationship to the regulation of its expression.

RATIONALE FOR EXPERIMENTAL APPROACH

Clearly, a detailed and comprehensive genetic study involving most of the known *lon*$^-$ mutations would provide a better understanding of the molecular mechanism(s) by which the *lon*$^+$ gene controls the expression of such diverse processes as cell division coordination, regulation of capsular polysaccharide synthesis, polypeptide degradation and inheritance of plasmids and prophage. Predictions concerning the molecular basis of the regulatory activity of the *lon* gene cannot be made unless the genetic structure of this locus is defined. The presence of either a single gene and gene product or a number of closely linked genes coding for different gene products would permit more accurate predictions of the relationship between genetic structure and function. Models of *lon* action could then be proposed based on the findings of such genetic studies.

Because the nature of the *lon* gene product as well as some of its characteristic functions are now known, it would seem attractive to postulate the existence of only one gene which specifies the synthesis of one gene product. If such were the case, then an alternative model would need to be proposed to explain the presence of several defects in one gene product perhaps as a result of multiple lesions along a single cistron.

Data has been gathered that supports the contention that either *lon* is a single gene or that there exists a more complex genetic structural organization. Bush and Markovitz (20) reported that the different

degrees of UV sensitivity and mucoidy observed in different Lon$^-$ strains correlated with map position and Markovitz and Baker (114) showed that nonsense suppressors did not suppress all Lon$^-$ phenotypes coordinately. Donch and Greenberg (42) presented evidence for the presence of at least two adjacent genetic "blocks" at the lon locus which exhibited differential expression with respect to UV sensitivity in the absence of outside suppressors of lon. In addition, from genetic crosses in which P1 phage, previously grown on a non-mucoid, UV resistant (UV^R) strain (lon$^+$), was used to transduce a mucoid, UV sensitive (UV^S) (lon$^-$) strain to the wild type phenotype, UV^R, mucoid and UV^S, nonmucoid recombinants which failed to segregate either parental type were isolated (Falkinham, unpublished). The possible genetic separation of these two Lon$^-$ associated phenotypes strongly suggested the existence of at least two functional units or cistrons at this locus.

The Lon mutants described so far expressed the mucoidy and UV^S phenotypes to different degrees, probably due to the different genetic backgrounds in which they had been originally isolated.

Since no comprehensive study has been done in which detailed genetic analysis, involving most of the lon mutations already described, has been used to define the structure of this region; the present study was undertaken to determine if the lon defect was due to several mutations at different sites within one gene or whether it was the result of different mutations within different genes. In addition, a linkage map of the region was derived in an attempt to correlate map

position with the degree of expression of specific defects characteristic of the Lon phenotype.

To establish any legitimate comparisons among a collection of independently isolated *lon*$^-$ mutations, it was necessary to minimize differences in genetic backgrounds that could interfere with the expression of certain Lon phenotypes. For this purpose, a set of isogenic strains differing only at the *lon* locus was constructed. In P1 bacteriophage mediated generalized transduction, the frequency of crossing-over, therefore, of co-transduction, is proportional to distance (95,185). The precise order of the different *lon*$^-$ alleles was established by growing P1 on each Lon$^-$ mutant and utilizing the lysates thus produced to transduce a single *proC*$^-$, *purE*$^-$ *E. coli* K-12 strain. The position of each *lon*$^-$ allele relative to each other and to each one of the outside markers could then be determined. At the same time, the group of *lon*$^-$ isogenic strains was generated.

Cotransduction frequency determinations cannot be accurately made in instances where the distance between the markers measured is small (95,117), since recombination events within short distances are not independent of each other (95). Furthermore, marker effects and high negative interference could add to the difficulties of interpreting mapping data (134). A most rigorous method for establishing the site where a mutation is located involves the mapping of known point mutations against deletion mutants (7,102,140,150). In this way, the accuracy of mapping is increased since deletion mapping depends only

upon the appearance (or absence) of wild type transductants and not upon the frequencies of different classes of transductants (95). Attempts were made to generate a series of deletions in a primary F-prime strain carrying the lac to min E region on the plasmid by nitrous acid mutagenesis (3,54,150). The deleted strains could then be used in crosses with each isogenic lon$^-$ strain. Deletions extending beyond the lon$^-$ point mutation were not expected to yield Lon$^+$ recombinants.

If the relative position of each lon$^-$ allele is known and a gradient in the expression of the mucoid and UV^S phenotypes can be correlated with map position; then, variations in the expression of other Lon-associated characteristics would be expected to occur. The extent to which each Lon mutant expressed each phenotype was measured to determine whether every lon$^-$ allele conferred the same range of phenotypes to an Escherichia coli K-12 strain. If mutations that map towards one end of the lon locus exhibit a specific group of characteristics while mutations mapping at the opposite end have another set of phenotypes, it may be inferred that at least two distinct functional regions (domains) are present at this locus.

Bush and Markovtiz (20) stated that most known lon$^-$ mutations were of the nonsense type and at least one lon$^-$ mutation had been characterized as being of the frameshift type (155). In order to determine whether all lon$^-$ mutations were due to base-pair substitutions, point mutations or deletions, the frequency of spontaneous and chemically-induced reversion of each lon$^-$ mutation was estimated in the isogenic set of strains constructed.

A more refined way of addressing the problem of explaining the organization of the lon locus is by use of the genetic complementation test (55). The construction of stable merodiploids of bacterial cells with the introduction an F-prime factor carrying a specific mutation facilitates the study of inter-allelic and intra-allelic interactions (158). Patterns of dominance and recessiveness can also be determined in this way. The phenomenon of episomal dominance characteristic of some capR (lon) alleles, has been determined from such studies (124). Using P1 lysates of Lon$^-$ strains, an isogenic set was constructed by transduction in a proC$^-$ derivative of the primary F-prime strain χ573. This strain carries a deletion corresponding to the chromosomal DNA carried on the plasmid. Complementation studies were then performed between lon alleles by transferring the plasmids carrying the point mutations into F$^-$ lon$^-$ isogenic strains. To allow for differences in dominance patterns of some lon alleles (115) reciprocal arrangements (e.g. lon$^-$ on either plasmid or chromosome) were used. Initial measurement of complementation was done between those alleles showing the largest differences in phenotypes.

Attempts were made to isolate and identify new lon$^-$ suppressor mutations. The isolation of such suppressing strains has made possible the identification of several loci involved in the regulation of cell division and production of capsular polysaccharide (62,89,104,137). The production of "split" phenotypes, in which one Lon defect but not others is suppressed (See section A.5, Review of the Literature), has helped to

explain the molecular basis of *lon* gene action. The study of any novel pattern of suppression of one (or more) *lon*$^-$ defect(s) from which essential information regarding the mode of action of this locus could be derived, is of utmost importance.

MATERIALS AND METHODS

A. Bacterial Strains and Bacteriophages

The bacterial strains and bacteriophages used for this work are described in Table II. Strain MC118 was used as recipient for construction of the isogenic set of Lon$^-$ mutants. The strain χ573, a primary F-prime carrying the *lac* to *minE* region on the plasmid, was used to construct Lon$^-$ mutants with the mutations on the plasmid for the complementation studies.

A map of the *Escherichia coli* K-12 circular chromosome showing the positions of relevant markers and the genes included in the F-prime donor is shown in Fig. 4. A detailed map of the region of the chromosome from min 8 to min 11 is shown in Fig. 5.

Bacteriophages MS2, T6 and P1L4 were from the collection of R. Curtiss III. Bacteriophage P1CM*c1r100* was obtained from J. Rosner. The lambda bacteriophage derivatives λcI857(0ts) and λcI857 were obtained from S. Gottesman.

B. Media and Buffers

Bacteria were routinely grown in Pennasay Broth (PB; Antibiotic Medium No. 3, Difco). PB solidified with 1.5% (w/v) agar (PBA) was used for strain purification and viable counts.

Luria's complete medium (LB; Bertani, 8) was used in growth of bacteria for preparation and assaying of phage lysates and in conjugation experiments. LBM was LB with $MgSO_4$ added to a final concentration of 0.01M. LB solidified with 1.5% (w/v) agar was used in antibiotic susceptibility tests. LBCM plates contained 20 μg of

Table II. Bacterial Strains[1]

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
A. *Escherichia coli* K-12				
M6	HfrP01	*thi-1, rel-1, lon-10, lacI22*, λ^-	Thi^-, UV^S, Muc, Lac^-, λ^S	A. Markovitz (113)
χA21		*recA::Tn10*	Tc^R	From S. Gottesman
JF50	F^-	*thr-16, lacZ76, tsx63, purE41, supE42, pyrF30, his-53, strA97*	UV^S, Muc, Est^-, His^-, $T6^R$	J.O. Falkinham (52)
χ59	F^-	*thr-1, leu-1, lacY1*, λ^- *thi-1*	$T1^R$, $T6^S$, Sm^S, Thr^-, Leu^-, Thi^-, Lac^-, λ^S	J. Weigle
MC102	F^-	*leu-6, thi-1, trpE38, purE42, lon-9(capR9), lacY1, galK2, mtl-1, xyl-5, ara-14, str-109 azi-6, tonA23, tsx-67*, λ^- *supE44(?)*	$T1^R$, $T6^S$, λ^S, Sm^R, UV^S, Muc.	A. Markovitz (113)

[1]All allele designations are those used by the Coli Genetic Stock Center (5,37).

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
MC118	F^-	leu-6,thi-1,proC34, purE42,trpE38,his-81, lacZ55,ara-14,xyl5, mtl-1,man-3,str-109, azi-6,tonA23,tsx-67, λ^-,$P1^{imm}$,supE44(?)	UV^R,Rou,$T1^R$,$T6^R$,λ^S $P1^S$(?)	A. Markovitz (114)
CBK130	F^-	proC::Tn5	Thy^-,Pro^-,Km^R	From C. Berg
PAM153	F^-	thi-1,lon-21,pyrD36, galK30,strA129	Ura^-,Thi^-,Sm^R,UV^S, Muc	Johnson and Greenberg (89)
χ289	F^-	Prototrophic supE42, λ^-	λ^S,$T4^R$,P1.L4^S	R. Curtiss (33)
ATC401	F^-	$purE^+$,MC102		P1(χ289), to MC102, select Ade^+, Torres-Cabassa
PAM401	HfrPO2A	his-81,metB1,relA1, lon-20	His^-,Met^-,UV^S,Muc	Greenberg, via B. Bachman
χ573	F'FORF4	O-lac-purE F/Δ, lac-purE,serA2,λ^-	Sm^S,Ser^-,λ^S,$T3^R$,$T6^S$	R. Curtiss
ATC802	F^-	leu^+,MC118	Thi^-,Pro^-,His^-,Ade^-, Sm^R	From EMS Mutagenesis Torres-Cabassa, this work

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
χ833	F^-	thr-16,lacZ76,proC24, tsx-63,purE41,supE42, pyrF30,his-53,strA97, xyl-14,cyc-A1,cycB2, supE42	$T6^R$,$T4^S$,λ^{++S},λvir2^S, P1CM^S	R. Curtiss
NI850	HfrH	(tsx::λcI857)/λ	$T6^R$	From S. Gottesman
AB1157	F^-	thr-1,leu-6,thi-1, argE3,his-4,proA2, lacY1,galK2,mtl-1, xyl-5,ara-14,strA31, tsx-33,λ^-,supE44	UV^R,Rou,Sm^R,$T6^R$,λ^S	DeWitt and Adelberg, via B. Bachman
AB1896	F^-	thr-1,leu-6,thi-7, argE3,his-4,proA2, uvrA6,lon-6,lacY1, galK2,mtl-1,xyl-5, ara-14,str-31,tsx-33, λ^-,supE44	UV^S,Muc,Sm^R,λ^S,$T6^R$	P. Howard-Flanders
AB1897	F^-	thr-1,leu-6,thi-1, argE3,his-4,proA2, uvrB5,lon-7,lacY1, galK2,mtl-1,xyl-5, ara-14,str-31,tsx-33, λ^-,supE44	UV^S,Muc,Sm^R,$T6^R$,λ^S	P. Howard-Flanders

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
AB1899 (CGSC #1899)	F^-	thr-1,leu-6,thi-1, argE3,his-4,proA2, lon-1,lacY1,galK2, mtl-1,xyl-5,ara-14, str-31,tsx-33,λ^-, supE44,sul(?)	UV^S,Muc,Sm^R,$T6^R$,λ^S	P. Howard-Flanders et al. (78)
ATC2000*	F'	O-lac···purEF/ATC134	UV^R,Rou	ATC200 x ATC134, select Ade^+
ATC2001*	F'	O-lac··lon-9···purE F/ATC130	UV^S,Muc	ATC302 x ATC130, select Ade^+
ATC2002*	F'	O-lac··lon-9···purE F/ATC132	UV^S,Rou	ATC302 x ATC132, select Ade^+
ATC2003*	F'	O-lac···lon-9···purE F/ATC134	UV^S,Muc	ATC302 x ATC134, select Ade^+
ATC2004*	F'	O-lac···lon-1···purE F/ATC130	UV^R,Muc	ATC317 x ATC130, select Ade^+
ATC2005*	F'	O-lac···lon-1···purE F/ATC131	UV^R,Muc	ATC317 x ATC131, select Ade^+

*Merodiploids constructed for this work.

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
ATC2006*	F'	O-lac···lon-1···purE F/ATC134	UV^R,Rou	ATC317 x ATC134, select Ade^+
SG4008	F^-	lon-r1,lac,str	UV^S,Muc,Sm^R,Su^o	From S. Gottesman
SG4009	F^-	lon-t2,lac,str	UV^S,Muc,Lac^-,Sm^o,Sm^R	From S. Gottesman
SG13109	F^-	sulA,leu::Tn10,strA	His^-,Sul^-,Leu^-,Sm^R,Tc^R	From G. Gottesman
SG13110	F^-	pyrD,sulB,strA	ura^-,Sul^-,Sm^R,His^-	From S. Gottesman
SG13022	F^-	pyrD,leu::Tn10,strA lonΔ-100	UV^S,Muc,ura^-,Leu^-,Sm^R, Tc^R	From S. Gottesman
B. Isogenic $proC^+$ lon^{-a}				
ATC100	F^-	lon-10	UV^S,Muc,Sm^R,$T6^S$	Torres-Cabassa, this work
ATC101	F^-	lon-300	UV^S,Muc,Sm^R,$T6^R$	Torres-Cabassa, this work
ATC102	F^-	lon-9	UV^S,Muc,Sm^R,$T6^R$	Torres-Cabassa, this work

[a]All strains derived from MC118.

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
ATC103	F^-	lon-21	UV^S,Muc,Sm^R,$T6^R$	Torres-Cabassa, this work
ATC104	F^-	lon-20	UV^S,Muc,Sm^R,$T6^S$	Torres-Cabassa, this work
ATC115	F^-	lon-6	UV^S,Muc,Sm^R,$T6^R$	Torres-Cabassa, this work
ATC116	F^-	lon-7	UV^S,Muc,Sm^R,$T6^R$	Torres-Cabassa, this work
ATC117	F^-	lon-1	UV^S,Muc,Sm^R,$T6^R$	Torres-Cabassa, this work
ATC120	F^-	lon-r1	UV^S,Muc,Sm^R	Torres-Cabassa, this work
ATC121	F^-	lon-t2	UV^S,Muc,$T6^S$,Sm^R	Torres-Cabassa, this work
ATC130	F^-	lon-9	UV^S,Muc,Sm^R	Torres-Cabassa, this work
ATC131	F^-	lon-9A	UV^R,Muc,Sm^R	Torres-Cabassa, this work

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
ATC132	F^-	*lon-9B*	UV^S,Rou,Sm^R	Torres-Cabassa, this work
ATC150	F^-	*lon-1*	UV^S,Muc,Sm^R	Torres-Cabassa, this work
ATC151	F^-	*lon-1A*	UV^R,Muc,Sm^R	Torres-Cabassa, this work
ATC152	F^-	*lon-1B*	UV^S,Rou,Sm^R	Torres-Cabassa, this work
ATC125	F'ORF4	0-*lac•••proC::Tn5•••purE* F/Δ*lac•••purE* χ573	Pro^-,Ade^+,Ser^-,$T6^S$, Sm^S	Torres-Cabassa, this work
C. *Isogenic F' $proC^+$ lon^{-b}*				
ATC28	F'	*$purE^+$*,*lon-Δ328*,χ573	UV^S,Muc,$T6^S$,Sm^S	Nitrous acid mutagenesis, this work
ATC300	F'	*lon-10*	Sm^S,$T6^S$,Pro^+,Ade^+, $Lon^!$	Torres-Cabassa, this work
ATC301	F'	*lon-300*	Sm^S,$T6^R$,Pro^+,Ade^+, Lon^-	Torres-Cabassa, this work

[b]All strains derived from strain ATC125.

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
ATC302	F'	lon-9	Sm^S,$T6^R$,Pro^+,Ade^+, Lon^-	Torres-Cabassa, this work
ATC303	F'	lon-21	Sm^S,$T6^R$,Pro^+,Ade^+, Lon^-	Torres-Cabassa, this work
ATC304	F'	lon-20	Sm^S,Lon^-,Pro^+,Ade^+	Torres-Cabassa, this work
ATC315	F'	lon-6	Sm^S,$T6^S$,Lon^-,Pro^+, Ade^+	Torres-Cabassa, this work
ATC316	F'	lon-7	Sm^S,$T6^S$,Lon^-,Pro^+, Ade^+	Torres-Cabassa, this work
ATC317	F'	lon-1	Sm^S,$T6^R$,Lon^-,Pro^+, Ade^+	Torres-Cabassa, this work
ATC320	F'	lon-r1	Sm^S,$T6^S$,Lon^-,Pro^+, Ade^+	Torres-Cabassa, this work
ATC321	F'	lon-t2	Sm^S,$T6^S$,Lon^-,Pro^+, Ade^+	Torres-Cabassa, this work

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
D. Isogenic lon$^-$ recA$^-$[c]				
ATC1100	F$^-$	recA::Tn10 ATC100	Sm^R,T6^S,Lon$^-$,Tet^R, RecA$^-$	Torres-Cabassa, this work
ATC1101	F$^-$	recA::Tn10 ATC101	Sm^R,T6^R,Lon$^-$,Tet^R, RecA$^-$	Torres-Cabassa, this work
ATC1103	F$^-$	recA::Tn10 ATC103	Sm^R,T6^R,Lon$^-$,Tet^R, RecA$^-$	Torres-Cabassa, this work
ATC1104	F$^-$	recA::Tn10 ATC104	Sm^R,T6^S,Lon$^-$,Tet^R, RecA$^-$	Torres-Cabassa, this work
ATC1115	F$^-$	recA::Tn10 ATC115	Sm^R,T6^R,Lon$^-$,Tet^R, RecA$^-$	Torres-Cabassa, this work
ATC1116	F$^-$	recA::Tn10 ATC116	Sm^R,T6^R,Lon$^-$,Tet^R, RecA$^-$	Torres-Cabassa, this work
ATC1120	F$^-$	recA::Tn10 ATC120	Sm^R,Lon$^-$,Tet^R,RecA$^-$	Torres-Cabassa, this work
ATC1121	F$^-$	recA::Tn10 ATC121	Sm^R,T6^S,Tet^R,Lon$^-$ RecA$^-$	Torres-Cabassa, this work

[c] P1CMclr100 grown on strain χA21 was used to infect Lon$^-$ isogenic strains and selection made for tetracycline resistance (Tc^R).

Table II. (Continued)

STRAIN DESIGNATION	MATING TYPE	GENOTYPE OR ALLELE	RELEVANT PHENOTYPE	REFERENCE, SOURCE OF DERIVATION STEPS
ATC1130	F^-	recA::Tn10 ATC130	Sm^R,UV^S,Muc,Tet^R, $RecA^-$	Torres-Cabassa, this work
ATC1131	F^-	recA::Tn10 ATC131	Sm^R,UV^S,Muc,Tet^R, $RecA^-$	Torres-Cabassa, this work
ATC1132	F^-	recA::Tn10 ATC132	Sm^R,UV^S,Rou,Tet^R, $RecA^-$	Torres-Cabassa, this work
ATC1150	F^-	recA::Tn10 ATC150	Sm^R,UV^S,Muc,Tet^R, $RecA^-$	Torres-Cabassa, this work
ATC1151	F^-	recA::Tn10 ATC151	Sm^R,UV^S,Muc,Tet^R, $RecA^-$	Torres-Cabassa, this work
ATC1152	F^-	recA::Tn10 ACT152	Sm^R,UV^S,Rou,Tet^R, $RecA^-$	Torres-Cabassa, this work

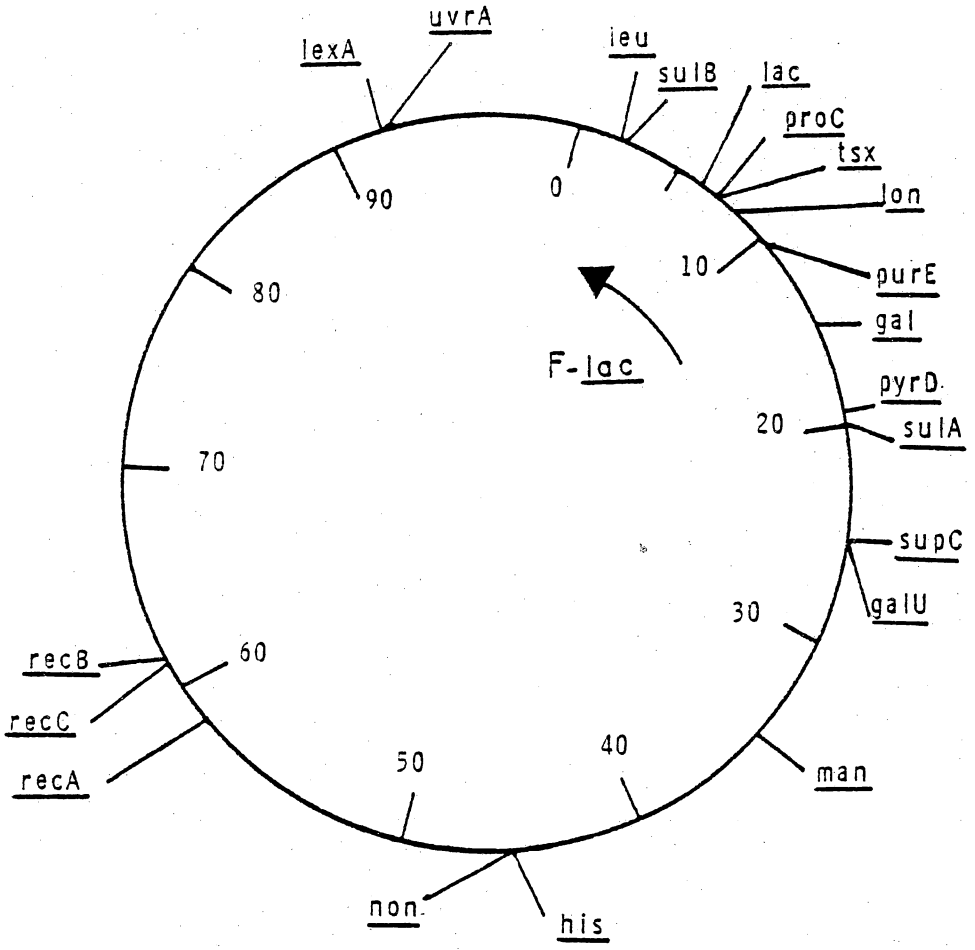

Figure 4. Circular chromosome map of Escherichia coli K-12. Adapted from reference 5.

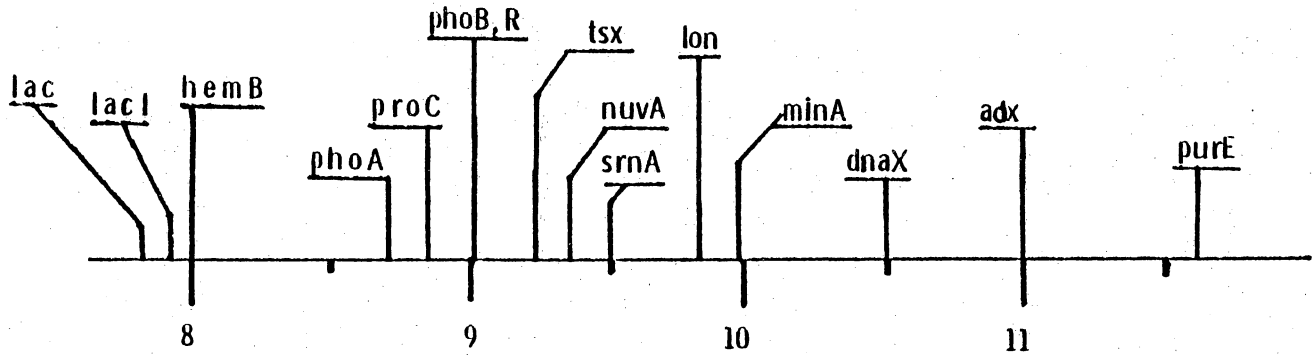

Figure 5. Map of the region from min 8 to min 11 on the Escherichia coli K-12 chromosome map showing the relative position of genes relevant to this work (5).

chloramphenicol (Cm) per ml. LBTc plates contained 20 μg of tetracycline (Tc) per ml. Luria's Broth with calcium (LC) containing 0.002M $CaCL_2$, 0.5% (w/v) glucose and 10 μg of thymine per ml was solidified with 1.0% (w/v) agar for plates and 0.7% (w/v) agar for overlays (LC top agar).

Yeast Extract Tryptone broth (YET; 78) 2.2% agar was used to determine viable counts in survival experiments.

Lambda (λ) agar contained 10 g of tryptone, 5 g of NaCl per liter, 0.2% (w/v) maltose, 0.01 M $MgSO_4$, 10 μg of vitamin B1 per ml and 1.0% (w/v) agar. Lambda soft agar had the same composition but it contained 0.65% agar.

Fermentator indicator medium was MacConkey agar (Difco) supplemented with 1.0% galactose (Mac-gal). EMBO plates (35) were supplemented with 5 g Yeast Extract and 5 g of NaCl per liter and were used for phage sensitivity tests.

Minimal media were that of Curtiss, et al. (33). Minimal broth (MB) and minimal agar (MA) were supplemented with amino acids, purines, pyrimidines and vitamins at optimal concentrations, as described by Curtiss, et al. (34) and contained 0.2 to 0.5% (w/v) carbon source. When specified, kanamycin sulfate (Km) or streptomycin sulfate (Sm) were added to a final concentration of 100 μg per ml.

Bacteria and phage were diluted in Buffered Saline with Gelatin (BSG) containing 8.5 g NaCl, 0.3 g KH_2PO_4, 0.6 g Na_2HPO_4, and 10 ml of 1.0% (w/v) gelatin per liter (33) unless otherwise indicated.

C. Chemicals: 9-Aminoacridine (9AO), chloramphenicol (Cm), kanamycin sulfate (Km), nalidixic acid (NA), nitrofurantoin (NF), streptomycin sulfate (Sm) and tetracycline (Tc) were obtained from Sigma Chemical Co., St. Louis, MO; ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS) and sodium nitrite were obtained from Eastman Organic Chemicals, Rochester, N.Y.; N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was obtained from Aldrich Chemical Company Inc., Milwaukee, Wis.; ICR-191 was obtained from Polysciences, Inc., Warrington, PA; crystal violet (CV) was obtained from Difco Laboratories, Detroit, MI.

D. P1 Lysate Preparation:

1. Plate Lysate method. Plate lysates of either P1.L4 or P1CMc1r100 were prepared as described by Miller (124). Donor bacterial strains were grown at 37C in LB with shaking to 4 x 10^8 cfu per ml. To 3 ml of melted LC top agar containing 0.5 M $CaCl_2$ and cooled to 45C, 0.5 ml of the grown bacterial culture and 0.1 ml of suitable dilutions of the phage were added, gently mixed using a vortex mixer and poured over the surface of an LC agar plate prewarmed at 37C. The plates were incubated at 37C. To plates whose lawn contained approximately 10^4 plaques, after overnight incubation, five ml of LB were added to each plate and these refrigerated for at least 3 h. The broth was then removed with a pipette and placed in a glass centrifuge tube and the top agar layer scraped off the surface of the plate, added to the tube containing the broth and mixed vigorously in a vortex mixer to break all clumps. 0.05 ml $CHCl_3$ was then added to the lysate, and the mixture

left at room temperature for 10 min. This lysate was then centrifuged for 10 min at 6000 x G, the supernatant containing the phage collected and $MgSO_4$ added to a final contentration of 0.01 M.

2. Thermal Induction Method. Lysates from thermoinducible lysogens were prepared as described by Rosner (142). Lysogens were grown in LBM at 30C with shaking to 2 x 10^8 cfu per ml. The cultures were then shifted to a 42C standing water bath for 20 min, then transferred to a 37C water bath and incubated with shaking until lysis was evident (Usually from 2 to 3 h.). To the lysed culture 0.15 ml of $CHCl_3$ were then added and the culture incubated for an additional 15 min with shaking. The cell debris was then pelleted by centrifugation for 30 min at 3000 x G, the supernatant containing the phage collected and the lysate stored with refrigeration.

For lysates in which P1.L4 was used, at least three cycles of infection, lysis and phage collection were carried out for each bacterial donor strain. Each time, the new lysate was used as the donor for the successive cycle and the third sequential lysate used for transductions. This procedure ensured enhanced exclusion of genes from strain χ289 on which the P1.L4 stock is grown.

E. P1 Titer Assay

A phage-sensitive strain (Either χ833 for P1CMclr100 lysates or χ289 for P1.L4 lysates) was grown at 37C in LB with shaking to mid log phase. The culture was then pelleted by centrifugation at 5000 x g for 10 min and resuspended in 0.5 M $CaCl_2$. To 2.0 ml of melted LC top agar

at 45C, 0.1 ml of the phage sensitive strain and 0.1 ml of the appropriate dilution of the freshly harvested lysate, were added, gently mixed in a vortex mixer and poured on LC agar plates prewarmed at 37C. Following incubation for 14 h at 37C, plaque counts were made. Titer for P1.L4 lysates were usually about 10^8 to 10^9 P1 per ml after three lytic cycles on Lon^- strains whereas lysates prepared by heat induction of Lon^- P1CMc1r100 lysogens yielded about 10^{11} plaque forming units (pfu) per ml.

F. Derivation of P1CMc1r100 Lysogens

P1CMc1r100 lysogens were prepared using a modification of the method of Rosner (142). Bacteria were grown in LBM at 30C with shaking to about 2 x 10^8 cells per ml. The cultures were then iced and $CaCl_2$ added to a final concentration of 0.01 M. To 0.1 ml of cell suspension, phage was added to a multiplicity of infection (moi) between 0.1 to 0.5 phage per bacterium and the mixture incubated at 30C for 30 min. After this incubation period part of the mixture was diluted 10-fold in BSG and 0.1 ml of the undiluted and diluted samples spread on LBCM agar plates, which were incubated at 30C for 48 h.

Following incubation, chloramphenicol-resistant (Cm^R) clones were purified as follows. Ten Cm^R colonies from each potential lysogen were transferred to each of 2 LB plates by touching each colony with a sterile wooden toothpick and inoculating the fresh plates with a small patch (5 lysogens per plate; 50 patches per plate). The plates were incubated overnight, the first set at 30C while the second was at 40C.

Patches that showed poor growth at 40C, yet grew at 30C, were assumed to have originated from lysogenic clones in which lysis had been induced by incubation at high temperature. Patches on the LB plate incubated at 30C that corresponded to lysed patches at 40C were further purified by streaking once on an LB agar plate and incubating at 30C overnight. Individual colonies were subsequently tested for the presence of relevant markers.

G. Test for P1 Lysogens

Formation of P1 lysogens were determined as follows. The strain to be tested was streaked on a PBA plate to obtain isolated colonies. Fifteen colonies were then picked and patched to a PBA plate and an LC agar plate. Both plates were then incubated at 37C. After 2 h approximately, 2 ml of melted LC top agar with calcium were mixed with 0.1 ml of phage-sensitive bacteria grown to mid-log phase, harvested by centrifugation at 5000 g for 10 min and resuspended in 0.5 ml adsorption medium. This mixture was then poured on the LC master plate, allowed to solidify and then incubated overnight at 37C. Following this incubation period, zones of lysis indicated lysogenic clones. Those patches around which no lysis was observed were considered not to be lysogens. The corresponding patch on the PBA master plate was then tested further as follows.

Overnight cultures of a phage-sensitive strain and of the putative non-lysogenic transductants were diluted 1: 100 in 5 ml LB and grown to about 2×10^8 cells per ml. A few drops of $CHCl_3$ were then added to

each culture and these let stand at room temperature for 1 h. An overlay of 2 ml melted LC top agar with calcium containing 0.25 ml of a culture of χ289 was then poured onto the surface of a prewarmed LC agar plate and allowed to solidify. The $CHCl_3$-treated cultures were then centrifuged at 3000 x g for 30 min, the supernatants collected and spotted on the LC agar plate by touching a loopful of each to the surface of the plate and the spots allowed to air-dry. The plate was then incubated overnight at 40C. Areas of lysis were observed wherever a culture supernatant of a lysogen had been spotted.

Formation of P1CMc1r100 lysogens was tested by the temperature sensitivity method as described above in section F of Materials and Methods. All lysogens identified in this way were able to produce viable P1 phage upon thermal induction (Section D, Materials and Methods).

Immunity to either P1.L4, P1KM or P1CM was tested by cross streaking overnight bacterial cultures with the phage on EMBO agar plates as described by Curtiss (35). On this medium a dark red coloration beyond the junction of the two streaks indicates sensitivity to the phage.

H. Selection of P1 Cured Cells

Thermoinducible P1CMc1r100 lysogens were cured of the phage by streaking bacterial cultures grown in LB at 30C and incubating overnight at 42C. Isolated colonies were then tested for their immunity to P1 as described in section G above.

I. Transductions

1. Transductions with P1.L4. Thansduction recipients were grown in LB at 37C with shaking to about 3 x 10^8 cells per ml. Cells were than pelleted by centrifugation and resuspended in 1.0 ml of 1.0% tryptone. To a plastic centrifuge tube were added 0.5 ml of adsorption medium, 0.5 ml of bacterial suspension and 0.5 ml of the donor lysate diluted in BSG to give a multiplicity of infection (moi) between 0.1 and 1.0 phage per bacterium. The mixture was then incubated at 37C for 20 min after which 5.0 ml of BSG were added to reduce P1 infection. The mixture was then centrifuged at 6000 x g for 10 min, the pellet resuspended in 1.0 ml of BSG and 0.1 ml of undiluted sample spread on selective MA glucose plates. The plates were then incubated at 37C for 72 h.

2. Transductions with P1CMc1r100. Recipients for transductions were grown in LBM at 37C with shaking to about 2 x 10^8 cells per ml, the cultures iced and made 0.01 M $CaCl_2$. To 0.1 ml of culture, 0.1 ml of P1CMc1r100 grown on the appropriate donor was added at an moi between 0.1 and 0.5 phage per bacterium. The mixture was then incubated at 30C for 30 min, diluted 10-fold in BSG and 0.1 ml of undiluted or diluted suspension spread on LBCM or other suitable selective plates. Incubation temperature of the plates varied with the purpose of the transduction. Whenever lysogens were to be constructed, the plates were incubated at 30C for 48 h. If determination of cotransduction frequencies or genetic backcrosses was the objective, incubation was at

37C for 48 to 72 h. This was done to prevent the formation of P1CMc1r100 lysogens. In all instances, cotransduction frequencies of mucoidy with proC obtained by using either P1-L4 or P1CMc1r100 lysates did not differ significantly.

J. Screening for the Inheritance of the Lon$^-$ Phenotype

To screen for the inheritance of the Lon$^-$ phenotype among selected transductants the replica plating technique of Lederberg and Lederberg (103) was used. Transductant clones from each cross were purified and tested for their degree of resistance to irradiation with ultraviolet light (UV) and their ability to produce mucoid colonies on supplemented MA glucose plates at 30C.

Master plates containing either 50 or 100 patches per plate were prepared for each transductional cross as described in section F, Materials and Methods. A patch of strain MC102 was included as a Lon$^-$ control along with a patch of either strain MC118 or strain AB1157 as a Lon$^+$ control on each plate. After overnight incubation at 37C, the master plates were replicated to 2 MA and 4 YET agar plates. Sensitivity to UV was then tested as follows. The YET replica plates were placed under the UV source exposed for either 0, 60, 120 or 180 sec in the dark and then incubated at 37C for 12 h protected from the light. The UV source was a General Electric 8 watt germicidal lamp with a dose rate of 12 ergs per sec per mm^2 as determined with an Ultra-Violet Products, Inc. J225 short wavelength intensity meter. UV sensitivity was evidenced by poor growth of the patch following the incubation period compared to UV resistant controls.

Ability to produce mucoid colonies was scored as follows. One replica MA plate was incubated at 37C and the other at 30C. The incubation time needed for the expression of the mucoid phenotype in selected transductants was found to be temperature-dependent. Mucoid patches appeared after 48 h on plates incubated at 30C whereas at least 72 h were needed for the same patches to become mucoid on plates incubated at 37C. Patches that were found to be both mucoid and UV sensitive by the replica plating method were considered to have inherited the lon gene.

With the appearance in subsequent experiments of transductant classes with varying degrees of UV sensitivity intermediate between UV-resistant and UV-sensitive strains, the replica plating technique proved inefficient and other screening methods were adopted (See section L, Materials and Methods).

K. Determination of Sensitivity to Antibiotics and Alkylating Agents

Screening of isogenic Lon$^-$ strains for their sensitivity to antibiotics and other antibacterial agents was done by several methods. Initially the replica plating method (Section F, Materials and Methods) was used but it was soon discarded since results were inconsistent.

Spot Tests: Using a calibrated loop, 0.01 ml of a standing overnight culture of the tester strain was spotted on the surface of a series of LB agar plates containing increasing concentrations of NF, MMS and NA. The concentrations in the media varied depending on the particular agent being tested. The spots were then allowed to air-dry

and the plates incubated at 37C. Tests for NF sensitivity were done in the absence of light (red light) and the plates incubated in foil protected from light, at 37C. Incubation time depended on the concentration of the antibiotic tested. Following incubation, spots were scored for growth in comparison to Lon^+ and Lon^- controls.

Streak Tests: Stationary cultures of the strains to be tested were streaked on LB agar plates containing different concentrations of NF, MMS or NA. All experimental manipulations and scoring were as described for spot tests.

Antibiotic Resistance in Liquid Medium: Because in the preliminary antibiotic sensitivity screening tests described above some, isogenic Lon^- mutants showed slight differences in their susceptibilities to NF and MMS, a third screening procedure allowing for a more accurate quantitative determination, was employed. The minimal lethal dose (MLD) of NF and MMS was determined by the method of Venturini and Monti-Bragadin (172) with some modifications. Bacteria were grown overnight in either LB or MB and 0.05 ml used to inoculate 5.0 ml of the same medium in glass tubes containing appropriate concentrations of the antibiotic being tested. LB cultures were then incubated for 10 h while MB cultures were incubated for 20 h at 37C. The extent of inhibition was then determined by the change in optical density at 450 nm relative to that of untreated controls. The minimal lethal dose was the concentration that caused a 50% decrease in optical density compared to the untreated control.

Sensitivity to Crystal Violet: To determine crystal violet sensitivity, 0.1 ml of suitable dilutions of bacterial cultures grown to mid-log phage were spread on MA plates containing different concentrations of the dye. The plates were then incubated at 37C for 48 h and viable counts made.

L. Sensitivity to Ultraviolet Light

UV survival curves were done following the method of Howard-Flanders, et al. (78). Overnight cultures of the strains to be tested were diluted 1: 200 in YET broth and grown at 37C with shaking until mid-log phase. The cultures were then centrifuged at 6000 x g, washed twice and resuspended in the same volume of BSG and the entire volume poured into a glass petri dish. The cell suspension was then exposed to the UV source for 0, 15, 30, 45 and 60 sec while swirling, suitable dilutions made in BSG and 0.01 ml samples plated on YET agar plates (For irradiation procedure and post-irradiation manipulations see section J, Materials and Methods.) The plates were then incubated in foil in the dark at 37C for 12 h after which viable counts were made.

For rapid preliminary screening of UV-sensitive strains and more accurate identification of intermediate UV-sensitive classes of transductants the following method was adopted. With a 10 µl calibrated loop, equal volumes of fresh standing PB cultures grown at 37C were used to inoculate a YET agar plate with a 3 cm streak across its center (15 streaks per plate). One half of the plate was then covered with aluminum foil to protect one half of each streak while the other

remained exposed. The plate was then irradiated with UV (source dose rate as above) for 30 or 60 sec. Following a 12 h incubation period at 37C the streaks were examined. Growth of the UV irradiated half was compared with both the unirradiated half and Lon^+ and Lon^- controls.

M. Production of Excess Capsular Polysaccharide

1. Quantitative Determination of Nondialyzable Methylpentose. Determination of the amount of capsular polysaccharide as nondialyzable methylpentose was obtained with a modification of the method of Gayda, et al. (62). Overnight standing cultures grown at 37C in PB were subcultured by diluting 1: 50 in supplemented MB and incubating with shaking at 30C for either 24 or 48 h. The cultures were then transferred to glass test tubes, boiled in a water bath for 10 min to release exopolysaccharides, centrifuged at 3000 x g for 30 min and dialyzed overnight against distilled water in the cold.

Quantitative measurement of nondialyzable methylpentose was done as described by Dische and Shettles (40) as follows. To 1.0 ml of iced dialysate sample, 4.5 ml of sulfuric acid-water mixture (6 H_2SO_4 : 1 H_2O) were added, the mixture warmed up to 22C for 3 min, boiled for 10 min in a water bath and then cooled slowly in running tap water. To the cooled mixture 0.1 ml of a 3% (w/v) cysteine-hydrochloride solution was added and allowed to stand at room temperature for at least one h to develop the characteristic color for methylpentoses. For each reaction mixture the absorbance spectrum between 360 and 430 nm was determined with a Hitachi Model 2 Digital Spectrophotometer. Concentrations of

nondialyzable methylpentose were calculated after Dische and Shettles (40). L-fucose was used as a standard.

2. Effect of Plating Medium and Incubation Temperature On Capsular Polysaccharide Production. Standing cultures of Lon^- strains grown at either 30C or 37C were diluted in BSG and 0.1 ml of the appropriate dilutions spread on MA, PBA or LB agar plates to give between 50 and 100 colonies per plate. Complex agar plates were then incubated at 37C and 30C for 24 h. MA plates were incubated at 30C and 37C for 48 or 72 h. Mucoid colonies were scored following this incubation period by comparison to Lon^+ and Lon^- controls.

N. Effect of Nalidixic Acid (NA) on Cell Division

The effect of NA on cell division was measured using a modification of the method of Kantor and Deering (91). Cultures of the strains to be tested were grown to 2×10^8 cells per ml in either MB or YET broth at 37C. NA was then added to a final concentration of 50 µg per ml, samples taken at various times, dilutions made in BSG at room temperature and 0.1 ml samples spread on MA or YET agar plates. Colony counts were made following incubation of YET agar plates for 24 h and of MA plates for 72 h at 37C. Growth of cultures was also monitored by following the change in optical density at 450 nm.

O. Effect of Nitrofurantoin (NF) on Growth and Cell Division

Survival, as determined by colony forming ability of NF-treated cultures was measured as follows. Mid-log phase cultures grown in either MB or YET broth at 37C in 250 ml red Erlenmeyer flasks were

exposed to different NF concentrations. At 0, 80, 160 and 240 min, samples were collected, dilutions made in BSG, and 0.1 ml samples plated on MA or YET agar plates. Incubation periods and viable counts were performed as described in section K, Materials and Methods. All manipulations were done in the presence of red light and the plates incubated in the dark.

The effect of NF and nucleosides on growth of Lon^- strains was determined by using a modification of the method of Kirby, et al. (97). Cultures grown in MB at 37C, were diluted 1 : 10 in fresh MB and grown with shaking in 250 ml red Erlenmeyer flasks at 37C until an OD_{600} between .20 and .35 was reached. The cultures were then divided into 3 equal portions, centrifuged at 5000 g for 10 min, washed once in BSG and resuspended in the same volume of fresh MB with NF or when indicated, 100 µg of guanosine and cytidine per ml. The treated culture was further incubated at 37C with shaking for 5 h and growth followed by optical density determinations at 600 nm.

P. Complex Medium-Induced Killing

The effect of a nutritional shift-up on survival was determined after Gayda, et al. (62). Cultures of Lon^+ and Lon^- strains were grown to 2×10^8 cells per ml in MB, suitable dilutions made in BSG and 0.1 ml samples spread on MA and YET agar plates in duplicates. The viable count on the YET agar plates expressed as a fraction of the viable count on the MA plates indicated the extent of killing.

Q. Measurement of Deg Phenotype

The ability to degrade nonsense or missense polypeptides of Lon$^-$ strains was determined indirectly by measuring the efficiency of plating (e.o.p.) and burst sizes of the temperature sensitive lambda (λ) bacteriophage (λcI8570ts) as described by Gottesman and Zipser (70). Strains AB1157 (lon$^+$), MC118 (lon$^+$), MC102 (lon-9;capR9), AB1899 (lon-1) as well as some isogenic lon$^-$ strains derived from MC118, were grown in 10 ml LB at 37C with shaking to mid-log phase, harvested and washed once in BSG, a second time in 10 mM $MgCl_2$ and resuspended in the same volume of 10 mM $MgCl_2$. Cells were then starved for 60 min at 37C. Following incubation, 1.0 ml amounts of cell suspension were distributed in 13 x 100 mm glass tubes. The phage diluted in 10 mM $MgCl_2$, was added to the bacterial suspension at an moi between 0.05 and 0.1 phage per bacterium. After a preadsorption period of 15 min at 37C, 0.2 ml of the mixture were then added to 2 ml of melted λ top agar, cooled in 45C and the suspension poured onto prewarmed plates. The procedure was done in duplicate with one set of plates incubated overnight at 30C, while the other set was incubated at 40C for the same length of time. The efficiency of plating was calculated from the phage titer at 40C divided by the phage titer on a lon$^+$ strain at 30C.

Temperature-sensitive phage burst sizes were done as follows. Bacteria were grown in LBM at 37C to an OD_{600} of about 0.35 and the cultures shifted to a 40C standing water bath. Phage was then added to an moi of 0.1 phage per bacterium. Following 10 min of adsorption at

40C, the mixture was then incubated at 40C with shaking for an additional 2 to 2.5 h, a drop of $CHCl_3$ was added and the mixture centrifuged at 3000 x g for 30 min. The supernatant was collected and titered on strain χ59 (a permissive strain) at 30C. Burst size was determined as phage out/phage in.

R. Determination of the Ability of Lon$^-$ Strains to Form P1 Lysogens

Bacteria grown in LBM to mid-log phase were iced and $CaCl_2$ added to a final concentration of 0.005 M. To 0.1 ml of cells 0.1 ml of suitable dilutions of a P1CMclr100 (χ833) lysate were added at an moi of 0.5 phage per bacterium. The mixture was then incubated at 30C for 30 min. After adsorption, appropriate dilutions were spread on LB and LBCM agar plates which were then incubated at 30C for 48 h. The efficiency of P1 lysogenization was determined from the viable count on LBCM expressed as a fraction of the viable count on LB plates (Gottesman and Zipser, 70).

S. Filamentation

Qualitative determinations of the degree of filamentation of Lon$^-$ transductants of MC118 were done as follows. Cultures grown overnight were diluted 1: 100 in LB or YET broth and incubated with shaking at 37C to mid-log phase. NF or NA was then added to the appropriate final concentrations and the cultures incubated further with shaking at 37C. At specific time intervals a drop of each culture was examined under a microscope for the presence of filaments.

Filament formation after UV irradiation was determined by observation of cultures prepared as follows. Mid-log phase cultures

grown in 5 ml YET broth at 37C were centrifuged at 5000 x g for 10 min, washed once in BSG and resuspended in BSG at one-tenth the original volume. This suspension was then exposed to UV light for 24 sec. Three ml of BSG were then added to the treated suspension and the cells harvested by centrifugation at 5000 x g for 10 min. The pellet was resuspended in 5 ml of fresh YET broth and samples examined under a microscope at 1 h intervals to assess formation of filaments.

T. Reversion Analysis

Tests for reversion of Lon$^-$ mutants and presumptive *lon*$^-$ deletions were done as described by Miller (124). Two drops of undiluted overnight cultures of the strain to be tested were spread to dryness on each of 4 YET or LB agar plates containing either NF or MMS. A 1 cm sterile filter disk was placed at the center of each plate and 0.05 ml of one of the following mutagens was added: NTG (1 mg per ml (w/v)), 9A (1 mg per ml (w/v)), EMS (Undiluted) or MMS (1 : 10 dilution in sterile water) and ICR-191 (1 mg per ml (w/v)). Following a 72 h incubation period at 37C, the plates were scored for revertants. Untreated suspensions were used for measuring the rate of spontaneous reversion. Isolated revertant colonies were then tested for UV sensitivity and mucoidy. NF plates as well as all plates treated with ICR-191 were incubated protected from light. All manipulations were done in semi-dark conditions.

U. Deletion Isolation

1. Nitrous Acid Mutagenesis. Attempts to isolate a lon$^-$ deletion mutation on the plasmid (F'ORF-4) in strain χ573 using nitrous acid mutagenesis were done as described by Miller (124). Fifteen cultures of χ573 grown overnight in 5 ml MB at 37C were centrifuged for 30 min at 3000 x g, washed in the same volume of 0.01 M acetate buffer (pH 4.62) and the pellet resuspended in 1.0 ml of 0.05 M nitrous acid. The mixture was then incubated at 37C for 15 min and the reaction stopped with the addition of 5 ml of BSG. The cells were harvested by centrifugation, resuspended in 10 ml of LB and grown for 24 h at 37C. The saturated cultures were then diluted in BSG and 0.1 ml of the appropriate dilution plated on MA plates containing glucose, serine and proline and seeded with 10^8 T6 phage. The plates were incubated for 72 h at 37C and the T6^R mucoid survivors purified by streaking on the same selective medium lacking T6. UV sensitivity was determined as described in section J, Materials and Methods. The mutant clones were purified further by determining their sensitivity to the male-specific phage MS2 by cross streaking (See section G, Materials and Methods) and by testing their reversion ability as described in section T, Materials and Methods.

V. Complementation and Recombination Between lon$^-$ Mutations

1. Mating Procedure and Complementation. Complementation tests were done by employing the replica mating method as described by Miller (124). RecA$^-$ derivatives of the F$^-$ lon$^-$ isogenic set were constructed

by transducing the strains to Tc^R with P1CM*clr100* (χA21) (*recA::Tn10*) (21,56,99). PBA master plates containing patches of *lon*$^+$ and *lon*$^-$ F-prime donors (11 donors per plate) were prepared from fresh streak plates and incubated at 37C for 8 h. Onto each one of 2 selective plates containing either NF or MMS in the appropriate concentration, 5 drops of a fresh overnight culture of the F^- recipient were spread and allowed to air-dry. The donor master plate was then replicated onto the selective plate containing the recipient and incubated for the required time period (See section K, Materials and Methods). Because strains carrying a *lon*$^-$ mutation on the plasmid showed increased resistance to NF and MMS (possibly due to the χ573 genetic background or gene dosage), streptomycin sulfate was added to the plates as described in section B of Materials and Methods. This selectively inhibited growth of the Sm^S donor without affecting the Sm^R recipients and ensured recovery of recombinants only. Experimental conditions were as described previously in section T of Materials and Methods. Confluent patch growth indicated positive complementation.

2. *Recombination Between lon*$^-$ *Alleles*. Qualitative recombination measurements were performed by using the spot mating technique described by Miller (124). Recovery of Lon^+ recombinants from crosses between 2 *lon*$^-$ strains was measured as follows. Donors and recipients were grown overnight in 2 ml PB. Using a calibrated loop about 0.01 ml of each F^- $RecA^+$ Lon^- recipient was spotted on the surface of an MA plate lacking serine and adenine and allowed to air-dry. An equal volume of the F-

prime lon$^-$ donor was then spotted on top of each F$^-$ recipient, the spots allowed to dry and the plates incubated at 37C for 48 h. Donors required serine and all recipients were adenine auxotrophs. Consequently, in only those spots in which succesful transfer of the episome had occurred was growth observed. Recombinants were purified as described below.

3. Purification and Characterization of Merodiploids. Following incubation of the selective plates, recombinants or merodiploids from selected crosses were purified as follows. Using a sterile wooden stick the center of each spot showing growth was picked and resuspended in 0.05 ml BSG. The suspension was then streaked on the appropriate selective medium and incubated at 30C overnight. Isolated colonies were then patched onto MA plates and incubated at 30C for 48 h to allow for the expression of the mucoid phenotype. Fifteen mucoid and fifteen nonmucoid colonies isolated in this fashion were then tested for their UV-sensitivity by the streak plate method (Section L of Materials and Methods). UV resistant-nonmucoid clones were assumed to be either Lon$^+$ recombinants or merodiploids. To test for the presence of the plasmid each colony was screened for sensitivity to MS2 (Section U, Materials and Methods) and its ability to transfer Ade$^+$ to an adenine (purE$^-$) auxotroph. Selected merodiploid classes were then characterized for UV sensitivity and mucoidy as described in sections J and L of Materials and Methods.

RESULTS

A. Mapping Independently Isolated lon$^-$ Mutations and Construction of Isogenic Strains.

1. Transductional Mapping. In order to construct an isogenic set of strains differing only at the lon locus and simultaneously generate a linkage map for this region, bacteriophage P1.L4 was grown on each of 10 independently isolated Lon$^-$ mutants and the resulting lysates used to transduce strain MC118 (proC$^-$, lon$^+$, purE$^-$) to Pro$^+$, Lon$^-$. Although Lon$^-$ mutants show reduced efficiency of P1 adsorption (52, 165), sufficient titers were obtained for the transduction mapping experiments. At multiplicities of infection of approximately 0.5 phage per bacterium, between 200 and 2000 ProC$^+$ transductants per 10^9 bacteria plated were obtained from most lysates. Primary selection of ProC$^+$ Lon$^-$ transductants of strain MC118 was done on minimal plates lacking proline incubated at 37C. Under these conditions, mucoid Lon$^-$ colonies can be distinguished from rough Lon$^+$ colonies.

Between 100 and 500 colonies including mucoid and nonmucoid clones, from each transductional cross, were screened for the UV^S phenotype. The results are summarized in Table III. Among all Pro$^+$ transductants Lon$^+$ (UV^R, nonmucoid) and Lon$^-$ (UV^S, mucoid) classes were found. In addition, two unexpected phenotypic classes appeared: UV^R, mucoid (Class A) and UV^S, rough (Class B). Class B transductants were found at a higher frequency than those observed for the UV^R, mucoid and UV^S, mucoid (Lon$^-$) classes (Table III). Measurements of frequencies obtained varied and the values in Table III reflect the average for each cross.

Table III. Cotransduction of UV sensitivity and mucoidy with *proC*[a].

Donor Strain	Donor Genotype *pro*	Donor Genotype *lon*	ProC$^+$ Transductants[b] % UV^R Rou	% UV^S Rou	% UV^R Muc	% UV^S Muc	Percent Cotransduction of *proC lon*[c]
M6	+	10	80.9	1.7	13.8	3.4	18.9
JF50	+	300	80.5	15.5	3.0	1.0	19.5
MC102	+	9	86.9	8.0	0.6	4.5	13.1
PAM153	+	21	75.8	4.42	2.04	17.7	24.16
PAM401	+	20	92.1	6.0	1.39	0.5	7.9
AB1896	+	6	70.4	11.3	11.3	6.8	29.6
AB1897	+	7	67.1	28.5	3.4	1.0	34.2
AB1899	+	1	68.2	16.9	7.7	7.1	31.8
SG4008	+	r-1	87.5	6.2	11.7	2.06	19.96
SG4009	+	t-2	60.2	31.6	1.02	4.08	39.8

[a]Crosses between P1.L4 lysates of the donor strains and strain MC118 (*proC lon*$^+$ *purE*) were carried out as described in Materials and Methods.

[b]Transductants were selected by spreading on minimal plates lacking proline at 37°C. Upon secondary, screening mucoid and rough clones segregated four intermediate phenotypic classes: UV^R Rou = UV resistant; rough (Lon$^+$); UV^S Rou = UV sensitive rough (Class B); UV^R Muc = UV resistant Mucoid (Class A) and UV^S Muc = UV sensitive Mucoid (Lon$^-$).

[c]Intermediate classes are included in the *proC*$^+$ *lon*$^-$ class to estimate transduction frequencies of *lon*$^-$ with *proC*$^+$.

The lon$^-$ alleles fell into two distinct classes based upon cotransduction frequencies to proC. The first group of mutations were weakly linked to proC$^+$ and included the alleles lon-9, lon-10, lon-20, lon-300 and lon-rl. Their frequencies of cotransduction with proC were between 7.9 and 20%. The second group, which included the alleles lon-1, lon-6, lon-7, lon-21, and lon-t2, exhibited higher transduction frequencies with proC ranging between 24.3 and 39.8%. Previously reported data of cotransduction frequencies between lon$^-$ and proC varied from 8.0% for the lon-rl and lon-t2 alleles (70) and 23% for the lon-Δ100 mutation (70) and 29% for lon-1 (42) (See Section F, Review of the Literature).

The data shown in Table III are in agreement with observations made by Donch and Greenberg (42) concerning the existence of intermidiate (UV^R Muc, UV^S Rou) Lon$^-$ transductants. These lon$^-$ alleles which were weakly linked to proC were more UV resistant than those which mapped closer to proC, though both were more UV sensitive than the lon$^+$ parent and transductants. (See Section B.1. Results). Fig. 6 shows the relative map position of all 10 lon$^-$ alleles and their linkage to proC.

Direct determinations of cotransduction frequencies of the lon$^-$ alleles with purE$^+$ were made by screening the ProC$^+$ Lon$^-$ transductants for inheritance of the Ade$^+$ marker. Never were Lon$^-$ Ade$^+$ recombinants observed. It had been assumed that those lon$^-$ alleles weakly linked to proC would yield some Lon$^-$ Ade$^+$ transductants, albeit at low frequencies. This was expected because the lon and purE genes are

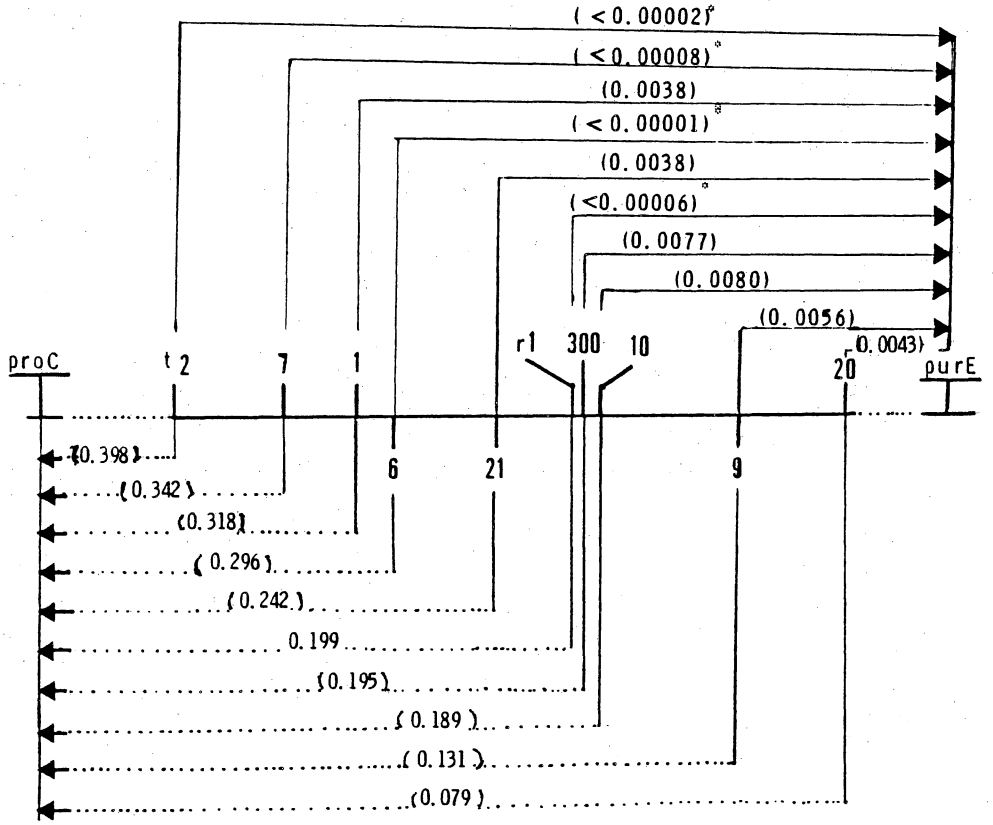

Figure 6. Linkage map of lon with proC and purE. Numbers above and below the line indicate allele designations. Numbers in parenthesis represent cotransduction frequencies. Arrows indicate the selected marker. (*) Accurate determinations of cotransduction frequency could not be made.

separated by a distance of 2 min on the Escherichia coli K-12 chromosome map. Correlation of frequencies for two loci with time units, determined from interrupted mating experiments for the same two markers, have shown that markers separated by a distance of 1.5 min or more are cotransduced 1% or less (124).

Attempts to measure directly the frequency of cotransduction of lon$^-$ mutations with purE$^+$ were made. Although higher multiplicites of infection were used (0.5 to 1 phage per bacterium) and about 300 to 500 transductants were obtained in each cross, no mucoid clones were observed. Preparation of a fourth and fifth P1.L4 lysate of each lon$^-$ donor failed to increase titers significantly. A new set of lysates was prepared by growing the temperature-sensitive mutant phage P1CMc1r100 on each Lon$^-$ donor. High titer lysates, on the order of 10^{11} pfu per ml, could be obtained from these lysogens by heat induction. Strain MC118 was then infected with each new lysate, with subsequent selection for Ade$^+$ transconjugants. The results are shown in Table IV and the relative position of those lon$^-$ alleles which produced Mucoid, Ade$^+$ recombinants are also indicated in Fig. 6. Of the six lon$^-$ alleles, which showed cotransduction frequencies of mucoidy with purE between 0.08 and 0.77%, (lon-1, lon-9, lon-10, lon-20, lon-21 and lon-300), five had previously been found to be weakly linked with proC. Transduction frequencies for the other four remaining lon$^-$ alleles could not be determined since no mucoid Ade$^+$ transductants were observed.

Table IV. Cotransduction of *lon* with *purE*[a]

lon allele	Number observed Ade$^+$	Mucoid	% Mucoid[b]
1	260	1	.38
6	578	0	<.001
7	121	0	<.008
9	714	4	.56
10	1237	1	.08
20	234	1	.43
21	776	3	.38
300	900	7	.77
r-1	165	0	<.006
t-2	414	0	<.002

[a] P1CM*c1r100* grown on the *lon*$^-$ strains was used to infect strain MC118. Selection was made for Lon$^-$ Ade$^+$ transductants. For recipient genotype refer to Table II.

[b] Number of mucoid colonies observed on the primary selection plates as a fraction of the total number of transductants obtained.

The data presented in Tables III and IV strongly suggest the existence of two "clusters" of *lon*$^-$ mutations, each found at opposite ends of the *lon* locus and conferring a different degree of expression of at least the UV sensitivity phenotype. This indicates that perhaps two functional regions (cistrons or domains) could be present at this site.

2. Segregation of Mucoid and Rough Colonies. The spontaneous segregation of nonmucoid clones from Lon$^-$ mucoid colonies at frequencies higher than those observed for spontaneous mutation had been noted previously (Falkinham, unpublished). Similar segregation of mucoid clones from *Pro*$^+$ rough transductants had been reported by Donch and Greenberg (42) and was interpreted as the result of segregation of partial heterogenotes in which the P1 transducing particle had persisted. I have observed segregation of Lon$^+$ (UV^R, Rou) clones from Lon$^-$ (UV^S, mucoid) transductants of MC118, particularly in crosses in which the P1 phage had been grown on a heavily mucoid donor.

If the transducing particle persists in the cell without recombining with the recipient chromosome, a pattern of unilinear inheritance and segregation will result (158). In addition, establishment of P1 lysogeny in the ProC$^+$ Lon$^-$ transductants would lead to immunity in subsequent crosses and possible interference in the expression of some Lon$^-$ associated phenotypes (142).

To investigate if segregation of mucoid and nonmucoid (rough) clones was responsible for the appearance of intermediate classes, segregation patterns of mucoid and non-mucoid colonies as well as of UV^S

and UV^R clones from representatives of all four phenotypic transductant classes in selected crosses were examined. Because the alleles from strains MC102 (*lon-9*) and AB1899 (*lon-1*) mapped at opposite ends of the *lon* locus and also differed in the expression of UV sensitivity and mucoidy, transductants obtained from crosses in which P1 grown on these two strains had been used as donor, were examined. Table V shows the result of the analyses done of Lon intermediate classes A and B obtained from crosses in which either P1(MC102) or P1(AB1899) was used to infect strain MC118 (*proC*$^-$, *lon*$^+$). The data indicates that even though intermediates do segregate, stable Class A and Class B Lon strains were found.

Segregation of UV^S, Pro^- and UV^R, Pro^- subclones was only observed in the UV^R, mucoid class. Generally, two additional cycles of testing were necessary before stable subclones could be obtained. Thus, it is apparent that in some cases the P1 particle failed to recombine with the recipient chromosome, though it remained in the cell long enough for the Lon^- phenotype to be expressed. The appearance of UV^R Pro^- subclones indicates that complete loss of the P1 particle occurred and thus those clones must have been unstable heterogenotes.

The appearance of UV^S, Pro^- subclones can be explained as follows. In the temporary partial heterogenote, the *lon-9* gene is dominant for mucoidy but not for UV sensitivity (115) and therefore a UV^R, mucoid phenotype results. If recombination occurs before the cell divides in such fashion that only the DNA segment coding for the *lon* gene is exchanged, then a UV^S, Pro^- cell could be produced.

Table V. Segregation Behavior of Lon Class A and Class B strains[1]

CROSS[2]	LON CLASS	STABLE[a]	UNSTABLE[b]			
			$UV^{S}Muc$	$UV^{S}Rou$	$UV^{S}Pro^{-}$	$UV^{R}Pro^{-}$
P1(MC102) x MC118	A(Pro^{+},$UV^{R}Muc$)	7/25	6/25	-	3/25	8/25
	B(Pro^{+},$UV^{S}Rou$)	9/25	-	16/25	-	-
P1(AB1899) x MC118	A(Pro^{+},$UV^{R}Muc$)	5/10	5/10	-	-	-
	B(Pro^{+},$UV^{S}Muc$)	5/10	-	5/10	-	-

[1]Colonies were streaked and tested for mucoidy and UV sensitivity. Three to five cycles of purification and testing were performed.

[2]For genotypes refer to Table II.

[a]Stable segregants were not examined further.

[b]Unstable subclones required at least 5 cycles of streaking and testing. Stable isolates were always obtained.

Similar results were obtained with P1(AB1899) lon-1 transductants although segregation of Pro^- subclones were not observed. Purified stable subclones of each class were selected, tested further for the presence of the P1 phage particle and saved for subsequent experiments.

3. Test for P1 Lysogeny and Curing. P1 lysogens of Escherichia coli K-12 are stable and cannot be cured of the phage (10,22,35,124,161, 162). P1.L4 is a mutant derivative of phage P1.kc that posseses a mutation in the cI repressor that prevents it from forming lysogens. Because we have observed that P1.L4 does form lysogens at somewhat low frequencies, all the isogenic Lon^- strains strains constructed were tested for the presence of P1.

Colonies from streak plates were picked and patched to complex medium, incubated at 37C for 3 h and the medium overlayed with melted top agar containing the P1.L4 sensitive strain χ289. Lysogens were formed in the isogenic set of Lon^- strains as indicated by small areas of lysis around the patches. When the intermediate UV^S, non-mucoid and UV^R, mucoid classes were tested, few or none of the colonies were found to be lysogens. The procedure was repeated twice and patches showing no lysis after the last cycle were considered to be cured of the phage. However, variation in colony morphology was still detected in most of the strains in subsequent experiments, indicating that some other mechanism(s), distinct from segregation of partial diploids, ws controlling the expression of the mucoid phenotype as previously noted by Falkinham (unpublished) and Donch and Greenberg (42).

B. Phenotypic Characterization of Each lon$^-$ Mutation in the Isogenic Set of Lon$^-$ Strains.

In addition to UV sensitivity and mucoidy, other genotypes have been associated with the lon$^-$ mutations. Table VI lists all known Lon$^-$ associated phenotypes described. If there exists a direct correspondence between map position and function of the lon gene product(s), as observed with sensitivity to UV irradiation, then a similar gradient of expression, and perhaps segregation, could be expected to occur in some or all Lon$^-$ associated phenotypes.

To establish whether all the lon$^-$ alleles conferred the same Lon phenotype when introduced into one genetic background, the isogenic set of Lon$^-$ strains derived from strain MC118 was screened for expression of most of the phenotypes included in Table VI. It was assumed that clustering of alleles would perhaps result in the generation of other "split" Lon phenotypes.

1. Sensitivity to Irradiation with Ultraviolet Light (UV) and Formation of Filaments. The degree of UV sensitivity for all isogenic Lon$^-$ strains as measured by survival frequencies after a dose of 360 ergs per mm^2 for strain MC118 and four isogenic set of strains are shown in Fig. 7.

The most marked increase in UV sensitivity was caused by the lon-9 allele, and the smallest increase in UV sensitivity occurred whenever either the lon-1 or lon-t2 allele was introduced into MC118. The results presented in Table VII are in good correspondence with the

Table VI. Lon Associated Phenotypes

Lon^- Phenotype	Reference
UV sensitivity on complex	78
UV resistance on minimal	78
Filamentation	78
Mucoidy	78,113
Nalidixic Acid Sensitivity (50 μg/ml)	91
Hydroxyurea Sensitivity (5 μg/ml)	91
Nitrofurantoin Sensitivity (2 μg/ml)	62
Crystal Violet sensitivity (1-5 μg/ml)	177
Methyl methane sulfonate sensitivity (0.025%)	89
Growth rate ($\underline{lon}^+ > \underline{lon}^-$ in complex, equal rates in minimal)	62
Complex medium-induced killing	62
Lambda lysogeny lowered	179
P1 lysogeny lowered	52,165
Lowered Polypeptide degradation (Deg^-)	70,154
Lowered Inheritance of F-plasmids (Est^-)	52
Segregation of mucoid and rough colonies	(Falkinham, Gottesman, unpublished)

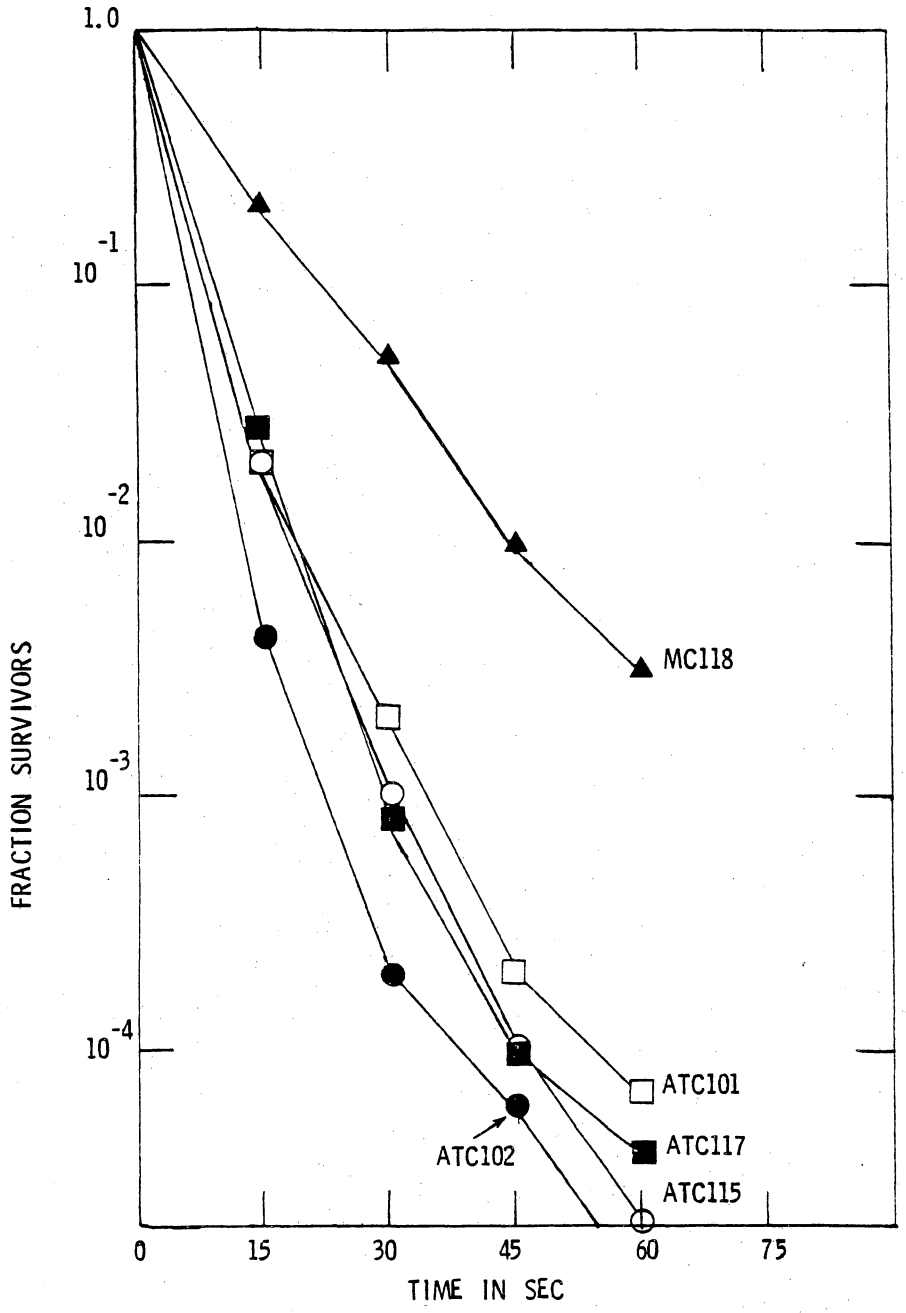

Figure 7. UV sensitivity of strain MC118 (lon$^+$) (▲) and four isogenic lon$^-$ strains. Lon alleles: lon-300 (□); lon-1 (■); lon-6 (○) and lon-9 (●).

Table VII. UV Survival Filamentation and Capsular Polysaccharide Overproduction of Lon⁻ Derivatives of Strain MC118.

Strain	lon allele	UV Survival[a]	Filamentation[b] Without UV	Filamentation[b] With UV %[1]	Filamentation[b] With UV Length[2]	Mucoidy[c] 30C	Mucoidy[c] 37C	Capsular Polysaccharide[d]
MC118	+	.043	-	-	.08	R	R	13.0
ATC100	10	.003	-	27.3	.42-1.43	+++	++	28.3
ATC101	300	.0021	+, long	6.3	.58-1.37	+++	+	35.2
ATC102	9	.0006	+, long	19.7	1.75-2.10	+++	++	32.5
ATC103	21	.0028	-	21.3	2.6	+	+	68.1
ATC104	20	.0012	-	1.0	.8-1.2	+	+	45.4
ATC115	6	.0083	-	5.1*	ND	+	+	44.3
ATC116	7	.0042	-	16.3	.8-2.0	+	+	41.2
ATC117	1	.013	-	12.1	.6-1.65	++	+	32.8
ATC120	r-1	.0085	-	66.0	.76-3.5	+	+	70.0
ATC121	t-2	.0122	-	1.8	.68-1.21	+	+	89.3

[a]UV survival expressed as the fraction of cells surviving after exposure to a dose of 12 ergs/mm^2/sec for 30 sec. Irradiated cells were plated on YET.

[b]Determined from microscopic observations 2 h post-irradiation.

[c]Determined from colony morphology on minimal plates at 37C.

[d]Capsular polysaccharide expressed as micrograms of nondialyzable methylpentose per ml of dialysate per unit of optical density was determined as described in Materials and Methods.

[1]Estimated percent of filaments is average of two observations.

[2]Expressed in micrometers.

*Estimated after 30 min of exposure; R = rough, (+) = mucoid.

linkage map obtained for the region since those *lon*$^-$ alleles which caused an increase in UV sensitivity greater than 20-fold, were those weakly linked with *proC*. Also, *lon*$^-$ alleles that resulted in only slight increases in UV sensitivity, had higher cotransduction frequencies with *proC*$^+$. Hence, it can be concluded that there exists a gradient in UV sensitivity at the *lon* locus determined by the map position of each allele within this region.

As indicated in Table VII, the extent of filamentation among isogenic Lon$^-$ strains varied; the length and fraction of filaments formed being directly related to their characteristic degree of UV sensitivity. Unirradiated cultures of strains ATC101 (*lon-300*) and ATC102 (*lon-9*) grown in YET broth, exhibited spontaneous filamentation, with about 50% of extremely long filaments after 4 h of incubation at 37C. These two strains were the most UV sensitive.

The data regarding the *lon-t2* allele is valuable for it shows the value of constructing the isogenic set. The original strain SG4009 was extremely UV sensitive, showing 99.9% killing with doses below 15 ergs per mm^2. When cultures were grown to an optical density at 450 nm of 0.1 to 0.2 in YET broth, about 90% of the cells were found as extremely long filaments (100 times normal size). This was not observed for the isogenic strain ATC121 which carries the same allele. Thus, the increased UV resistance when the allele is introduced into a different background, is most likely due to the elimination of a spontaneous filamentation defect in strain SG4009.

2. Production of Mucoid Colonies: Measurement of Nondialyzable Methylpentose. The ability to produce mucoid colonies was measured qualitatively for all isogenic Lon$^-$ strains, as well as for the lon$^+$ parent MC118. The strains were grown in ML until mid-log phase at either 30C or 37C and appropriate dilutions spread onto minimal complete plates which were then incubated at either 30C or 37C for 72 h. Alternatively, cultures were grown overnight, streaked onto PBA or MA plates in duplicates and one set of each incubated at 30C while the second was at 37C. All isogenic Lon$^-$ strains produced mucoid colonies at 30C after 48h. Mucoid colonies appeared after 72 h on plates incubated at 37C. During the first trials most of the colonies incubated at 37C appeared to be much less mucoid than those colonies found on plates incubated at 30C. Furthermore, when mucoid colonies appearing on NA plates incubated at 37C were picked and patched to the same medium and incubated at 30C none of the patches appeared to be mucoid. Neither the addition nor deletion of proline from the medium, nor changing the concentration of the carbon source, caused a change in the degree of mucoidy observed or in the disappearance of mucoid colonies during the replica plating procedure.

For this experiment, thymine had been added to the medium (40 µg/ml). Mucoidy was tested by repeating the procedure mentioned above and spreading the bacterial samples on MA plates containing 0,1,10,20 or 30 µg of thymine per ml. While colonies were smaller and appeared non-mucoid in plates containing thymine, even at low concentrations, Lon$^-$

colonies appeared larger and heavily mucoid at both 30C and 37C on the plates which lacked thymine.

A similar situation to that observed when thymine was lacking from the medium was subsequently noted in mating experiments where the medium contained thymine but adenine was lacking. In fact, a relationship between the degree of mucoidy observed and the sizes of nucleotide pools has been noted previously (104,105). In some non-mucoid lon-9, non-2 suppressed strains large quantities of GTP and UDP-glucuronic acid accumulate (104,105). Addition of thymine to the medium could cause a decrease in the degree of mucoidy for Lon^- strains probably by interfering with the biosynthesis of UDP-glucose, UDP-glucuronic acid and UDP-galactose, all components of the colanic acid produced in excess by Lon^- mutants.

The overproduction of colanic acid can be measured directly by determination of the amount of nondialyzable methylpentose produced by Lon^- strains since L-fucose is found as a sugar in this heteropolysaccharide. When Lon^+ and Lon^- cells were allowed to grow in supplemented ML at 30C for 72 h, all isogenic Lon^- strains, produced more nondialyzable methylpentose than did the Lon^+ parent. The results of these determinations are shown in Table VII. Although the strains were grown in ML containing 10 μg of thymine per ml, after 72 h incubation time, detectable quantities of methylpentose had been produced. The apparent high levels of sugars detected in the dialysate obtained from MC118 (lon^+) was due to the presence of heptoses and pentoses.

Strains carrying lon$^-$ alleles more closely linked to proC$^+$ produced weakly mucoid clones on MA plates, while isogenic strains with alleles weakly linked to proC$^+$ formed heavily mucoid colonies on the same medium. The mucoid appearance of the colonies produced by the isogenic Lon$^-$ strains showed no specific correlation with the amount of colanic acid produced measured as nondialyzable methylpentose. Surprisingly, strains that produced less colanic acid under the conditions tested, tended to map towards the middle of the locus. This could indicate that for some yet unknown reason, these mutations make their carriers more susceptible to inhibition by thymine. In spite of this unexpected result, it can be concluded that a gradient in the degree of mucoidy can also be correlated with map position at this locus. The relationship between the amount of colanic acid produced by the isogenic strains and their characteristic degrees of UV senstivity is shown in Fig. 8. With the exception of strains ATC100 (lon-10), ATC103 (lon-21) and ATC120 (lon-r1), increased production of colanic acid seemed to be related to a decrease in UV sensitivity.

3. Effect of Nalidixic Acid (NA) on Cell Division. The effects of NA on colony survival and formation of filaments in minimal and complex media were measured in the isogenic set of Lon$^-$ strains as well as in strains MC118 and MC102. The results are summarized in Table VIII and representative survival curves shown in Fig. 9. All cultures were grown to mid-log phase at 37C in both media and subsequently exposed to 50 µg NA per ml for 3 h. The data show that sensitivities to NA vary greatly

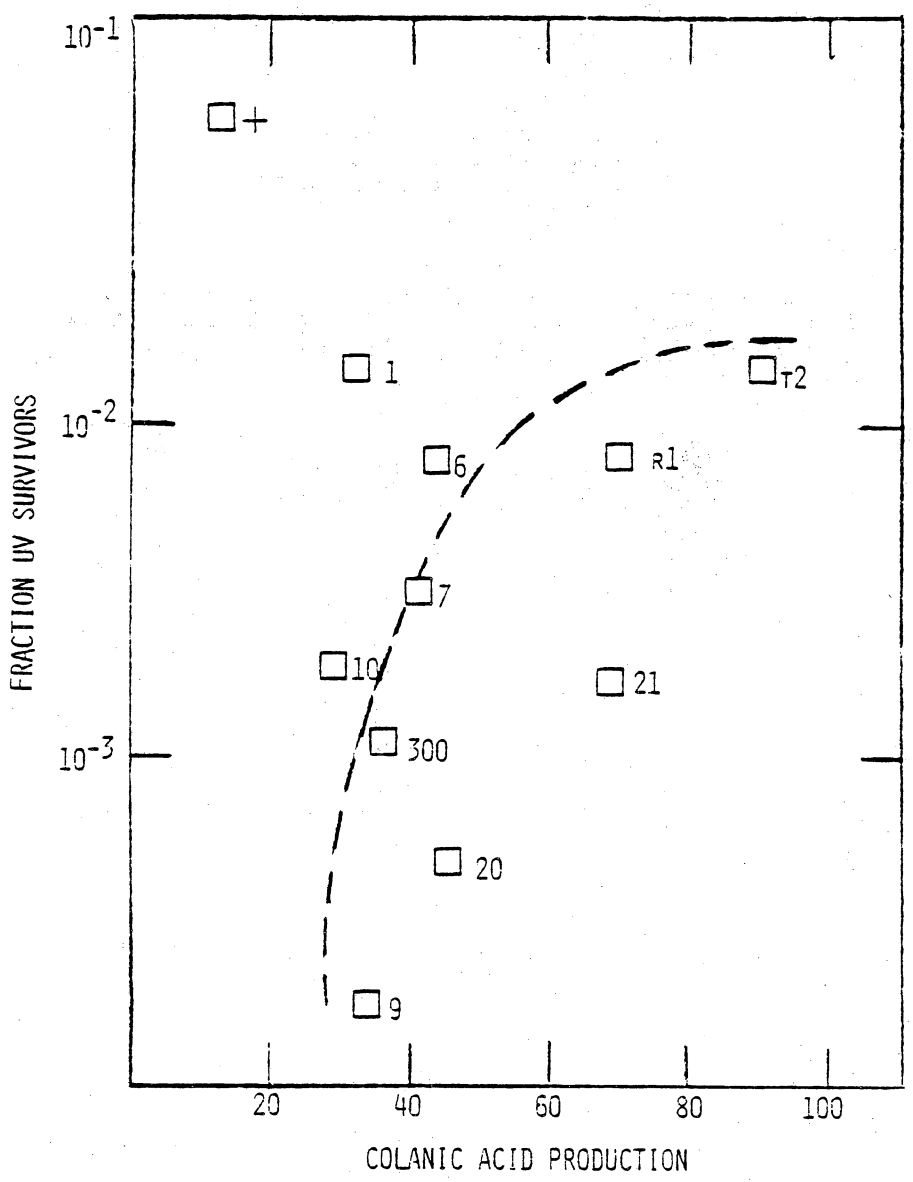

Figure 8. Relationship between the degree of mucoidy (determined as production of colanic acid) and UV^S (Table VII) of the isogenic Lon^- strains. Numbers are allele designations. + = MC118.

Table VIII. Effect of Nalidixic Acid on Cell Division of the Isogenic Lon⁻ strains.

Strain	*lon* allele	NA survival[a] Minimal	Complex
MC118	+	.36	.065
ATC100	10	.78	.38
ATC101	300	.38	.09
ATC102	9	.16	.01
ATC103	21	.38	.0026
ATC104	20	.26	.10
ATC115	6	.54	.16
ATC116	7	.90	.09
ATC117	1	.83	.005
ATC120	r-1	.75	.01
ATC121	t-2	.24	.24

[a]NA survival expressed as the fraction of cells surviving treatment with 50 μg of nalidixic acid per ml for 120 min in either minimal salts medium or YET broth.

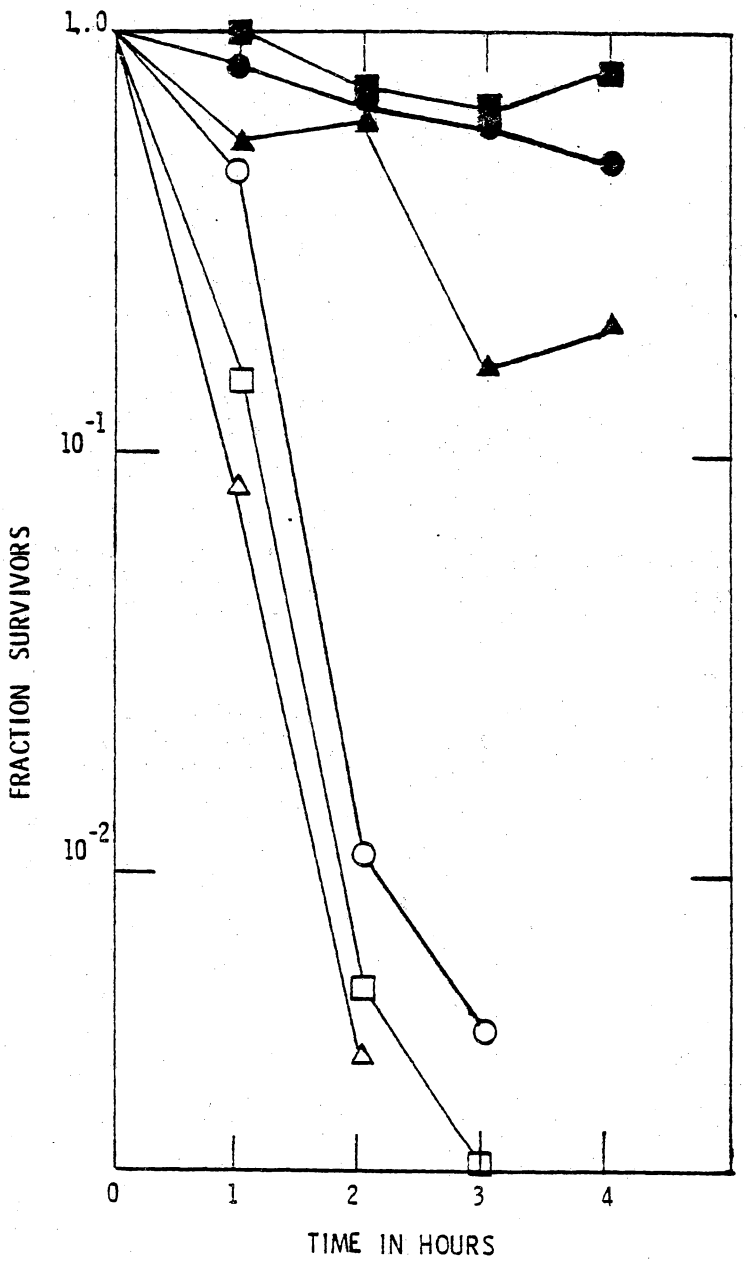

Figure 9. Comparison of nalidixic acid sensitivity in three isogenic Lon$^-$ strains. Closed symbols: cells grown in minimal medium. Open symbols: cells grown in YET. Strains: (■,□), ATC117 (lon-1); (●,○), ATC120 (lon-r-1) and (▲,△), ATC102 (lon-9).

among isogenic Lon$^-$ strain (Table VIII). Strains ATC102 (lon-9), ATC103 (lon-21), ATC117 (lon-1) and ATC120 (lon-r1) showed increased susceptibility to NA, compared to the lon$^+$ parent. Between a 6 and 25-fold increase in sensitivity to NA was observed for these strains. The remaining six isogenic Lon$^-$ strains registered a slight increase (2-5 fold) in NA resistance over that of the Lon$^+$ parent.

When cells grown in ML were exposed to the drug at the same final concentration, the surviving fraction of each Lon$^-$ strain after 2 h of exposure was found to be close to that of the lon$^+$ parent and differences in susceptibilities to NA among the isogenic strains were reduced. Nevertheless, some strains registered a slight increase in sensitivity to NA. In addition, with the exception of strains ATC100, ATC103 and ATC120, a relationship was found between the amount of colanic acid produced by each isogenic Lon$^-$ strain (Section B.2., Results; Table VII) and its degree of sensitivity to NA (Fig. 10); the more colanic acid, the higher survival to NA. This suggested a protective role of the capsular polysaccharide against the effects of the antibiotic.

Comparisons between treatments in two different growth media for each isogenic strain showed large differences in survival. Strains ATC102, ATC103, ATC117 and ATC120 showed lowered survival with a 17 to 160-fold decrease in the fraction of survivors observed for these strains when the cells were treated in complex medium, compared to their survival in minimal medium under the same conditions. On the other

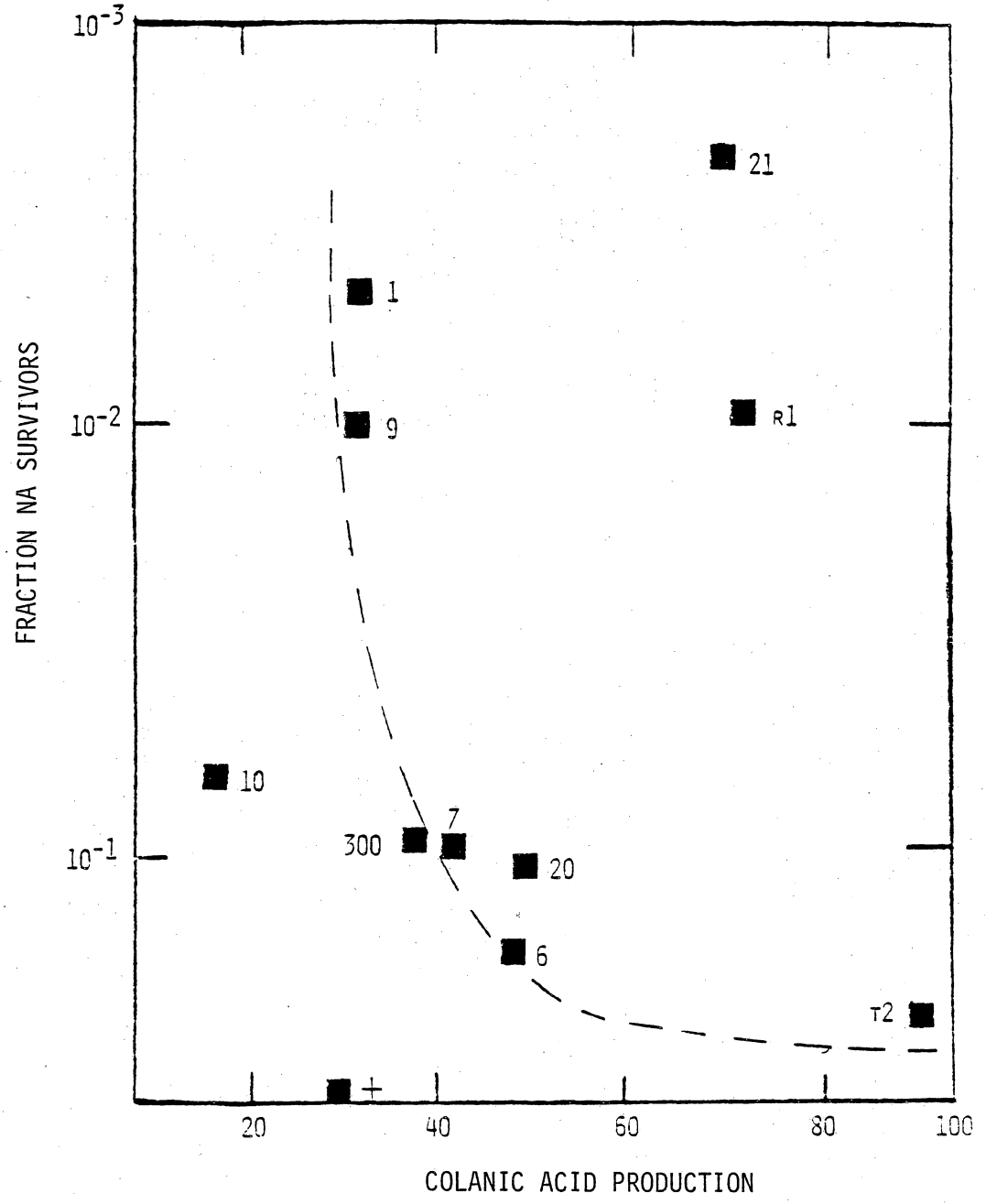

Figure 10. Relationship between the degree of mucoidy (colanic acid determined as nondialyzable methylpentose production) (Table VII) and the nalidixic acid sensitivity in complex medium (Table VIII) of Lon$^-$ isogenic strains. Numbers are allele designations. + = MC118.

hand, the remaining six isogenic strains showed a decrease in survival that varied between 2 and 10-fold when the same comparison was made.

Even though mass increase, as determined from the increase in optical density at 450 nm, was initially identical for Lon^+ and Lon^- strains, after 1 h of continuous exposure to NA, mass increase of treated Lon^+ cultures continued at a much slower rate than that observed for the control untreated culture (Fig. 11.B). By contrast the increase in optical density was the same in both, treated and untreated cultures of the Lon^- strains (Fig. 11.A). In the latter case, the fraction of viable cells in the treated culture decreased. Thus, the increase in optical density observed for Lon^- strains after treatment with NA is due to their increased degree of filamentation, as judged by microscopic inspection.

When differences in exposure treatment in the two different growth media are compared, the isogenic Lon^- strains can be classified into one of three groups on the basis of the observed shift in sensitivity to NA. Group I includes strains which show a drastic decrease in survival when exposed to NA in complex medium as compared to ML medium (lon-9, lon-21, lon-7, lon-300, lon-1 and lon-r1). Group II includes those Lon^- strains that show a slight improvement in survival in complex medium (lon-10, lon-6 and lon-20). Strains that show no significant change in their survival when treated with NA in both media are found in the third group (lon-t2). Kantor and Deering (91) reported that Lon^- mutants of Escherichia coli K-12 were more resistant to NA than the Lon^+ parent in

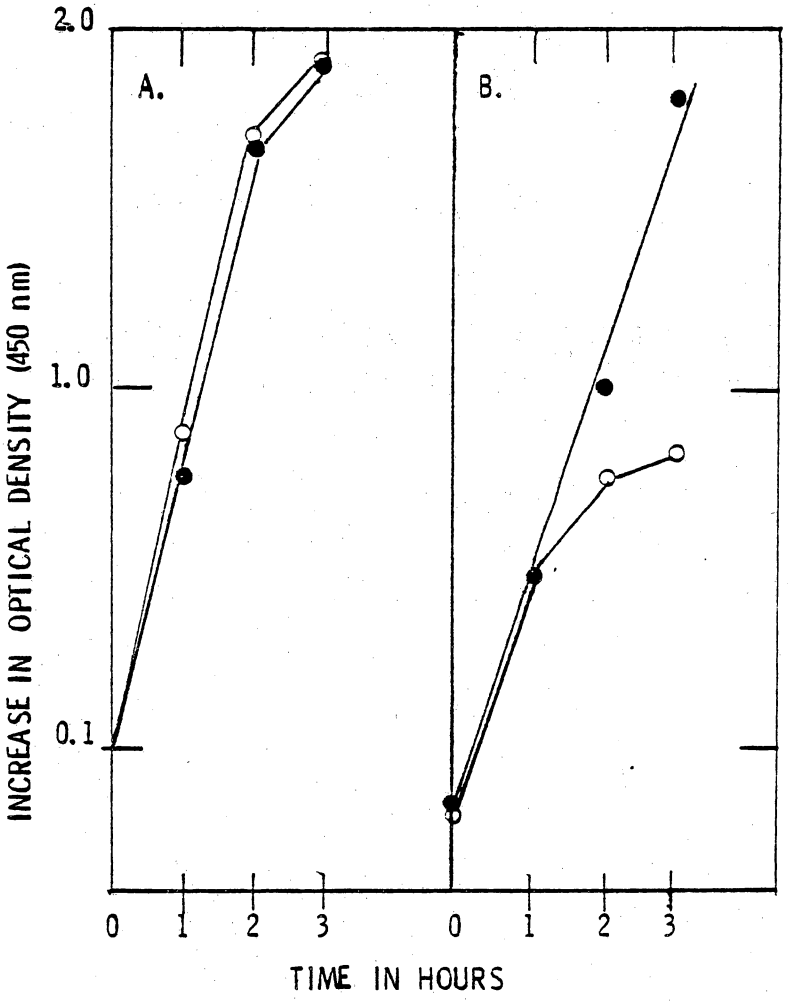

Figure 11. Effect of continuous exposure to nalidixic acid on $\underline{lon}^+$ and $\underline{lon}^-$ growth. A. Strain MC102 (*lon-9*). B. Strain MC118 ($\underline{lon}^+$). Symbols: ●, control cultures; ○, cultures treated with 50 μg nalidixic acid per ml.

minimal medium. The data presented in Tables VII and VIII show that such is the case with some Lon$^-$ strains investigated here. There seems to be no direct correlation between map position and the degree of NA sensitivity observed for the isogenic Lon$^-$ strains if their behavior in both media is considered.

4. Complex Medium-Induced Killing. When cultures of unirradiated capR9 (lon-9) cells grown in YET broth are examined under the microscope, numerous non-septate filaments are observed (2). Furthermore, capR9 (lon-9) cells are found to spontaneously form filaments when transferred from minimal to complex medium, with a reduction in colony forming ability (complex medium induced killing) accompanying this change (62).

To determine the extent of killing that such a nutritional shift-up would have on the other Lon$^-$ strains, cells were grown in ML to either mid-log or stationary phase, appropriate samples spread on minimal and complex plates and incubated at 37C. The results of these experiments are presented in Table IX. Increased complex medium induced killing was observed when cultures were in mid-log phase; with strain ATC102 (lon-9) showing the greatest decrease in survival (19%). All isogenic Lon$^-$ strains were found to be sensitive to the nutritional shift-up and when colonies growing on YET plates were resuspended in BSG and examined under the microscope, large numbers of filaments were observed in each case. Strains ATC100, ATC115, ATC116, ATC117 and ATC120 were found to be more resistant to killing regardless of the growth stage of the

Table IX. Complex Medium-induced Killing[a]

Strain	*lon* allele	Stationary[b] Phase	Mid-log[c] phase
MC118	+	1.13	2.00
MC102	9	.11	.29
ATC100	10	1.17	.64
ATC101	300	.91	.25
ATC102	9	.36	.19
ATC103	21	.96	.42
ATC104	20	.88	.64
ATC115	6	1.03	.93
ATC116	7	1.02	.75
ATC117	1	1.114	.35
ATC120	r-1	1.03	.77

[a]Extent of killing expressed as the plate count on YET divided by the plate count on minimal.

[b]Cells were grown overnight in minimal liquid medium at 37°C.

[c]Cells were grown to mid-log phase in minimal liquid medium at 37°C.

culture. Strain ATC117 ($lon-1$) was resistant to killing in the stationary phase but highly sensitive when in mid-log phase.

Even though no correspondence between map position of the *lon*$^-$ alleles and the degree of sensitivity to complex medium-induced killing was evident, those strains found to be slightly resistant to killing and also shown increased survival when exposed to NA. This suggests that the filamentation response observed after Lon$^-$ cells are exposed to either one of these two treatments is mediated by a common mechanism.

5. *Effect of Nitrofurantoin (NF) on Cell Division*. Lon$^-$ strains have been reported as being about 20-times more sensitive to the lethal effects of nitrofuran derivatives than Lon$^+$ strains (119). It is also known that nitrofurazone, in low concentrations, causes almost 100% filamentation of strain AB1899 (*lon-1*) (105) and thus mimics the effect of UV irradiation.

The effect of NF on cell division of all 10 Lon$^-$ strains was determined. Each strain was grown to mid-log phase in minimal medium at 37C and NF was then added at a final concentration of 5 μg per ml. Gayda, *et al*. (62) reported that Lon$^-$ strains were sensitive to 2 μg of NF per ml. All 10 isogenic, strains were found to be resistant at this NF concentration, but more sensitive than the *lon*$^+$ parent at NF concentrations between 5 and 10 μg per ml (Table X). This difference in sensitivity could be due to the MC118 genetic background.

The results of these experiments are shown in Table X and Fig. 12. From the survival values obtained, the isogenic strains clearly fell in

Table X. Nitrofurantoin and Crystal Violet Sensitivity of Isogenic Lon⁻ Strains.

Strain	lon allele	NF Sensitivity[a]	CV sensitivity[b]
MC118	+	2.40[1]	1.23
ATC100	10	0	NT
ATC101	300	2.77	.64
ATC102	9	.416[2]	.55
ATC103	21	.714	.69
ATC104	20	.166	.42
ATC115	6	5.25	1.00
ATC116	7	.106	.33
ATC117	1	5.38	.66
ATC120	r-1	1.58	.72
ATC121	t-2	4.00	.98

[a] NF sensitivity expressed as the fraction of surviving cells after treatment with 5 µg of nitrofurantoin per ml for 4.5 h in minimal medium.

[b] Surviving cell fraction after treatment with 20 µg crystal violet per ml in minimal medium for 90 min.

[1] Survival after 4 h. Average of 4 experiments.

[2] Survival after 4 h. NT = not tested.

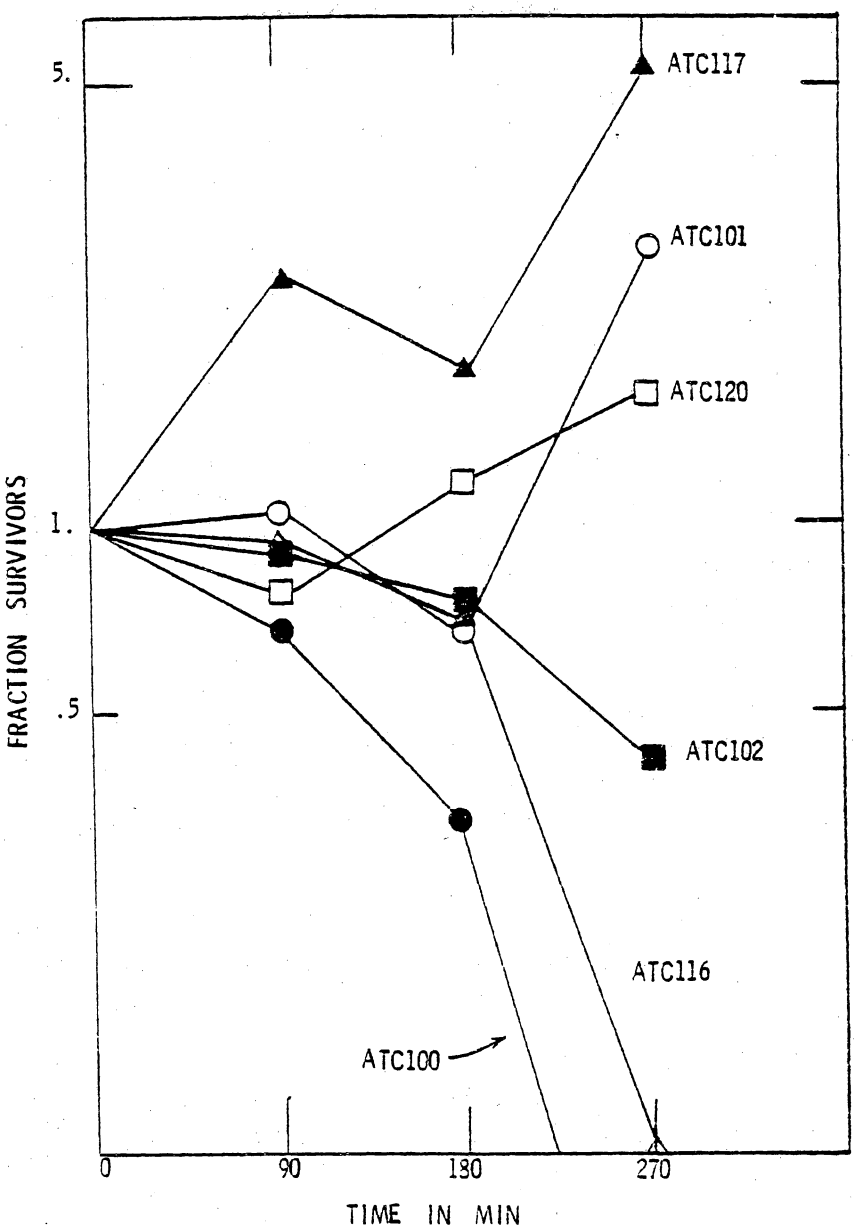

Figure 12. Effect of continuous exposure to nitrofurantoin on cell division of isogenic Lon⁻ strains. Cells were grown to mid-log phase in minimal medium. Nitrofurantoin was then added (50 µg/ml) and viable counts made every 90 min. Symbols: ▲, (lon-1); ■, (lon-9); ●, (lon-10); △, (lon-7); □, (lon-r1) and ○, (lon-300).

one of two groups. The more NF resistant (NF^R) strains were ATC101 (lon-300), ATC115 (lon-6), ATC117 (lon-1), ATC120 (lon-r1) and ATC121 (lon-t2). With the exception of lon-300, all these alleles map closer to $proC^+$ and confer more UV sensitivity compared to other lon^- alleles. This is in agreement with previous findings which indicated that resistance to NF develops in parallel with UV resistance (97). Furthermore, lon^- suppressed strains can be obtained by selecting NF^R clones that are also UV^R (62). Therefore, it seems that NF sensitivity is more closely associated with UV sensitivity than with other Lon^- associated defects (e.g., Mucoidy, NA sensitivity, Complex-medium induced killing).

Kirby, et al. (97) showed that simultaneous exposure of cells to NF and nucleosides resulted in normal cell division, although this was found not to occur with strain AB1899 (lon-1). Fig. 13 shows the result of an experiment carried out with strains AB1157 (lon^+) and MC102 (lon-9) in which cells were grown in ML with NF (5 μg/ml) or NF (5 μg/ml) plus guanosine and cytidine (100 μg per ml). The efficiency of NF in inhibiting growth of the Lon^- strains was evident since no increase in optical density was observed after 3 h of exposure. On the other hand, the Lon^+ strain recovered from inhibition after 1 h of exposure to the drug although the increase in optical density was lower and never reached that of the control cultures. Addition of nucleosides to NF treated cultures reversed the inhibitory effects of NF in both Lon^+ and Lon^- cells. A similar experiment done with strain AB1899 gave

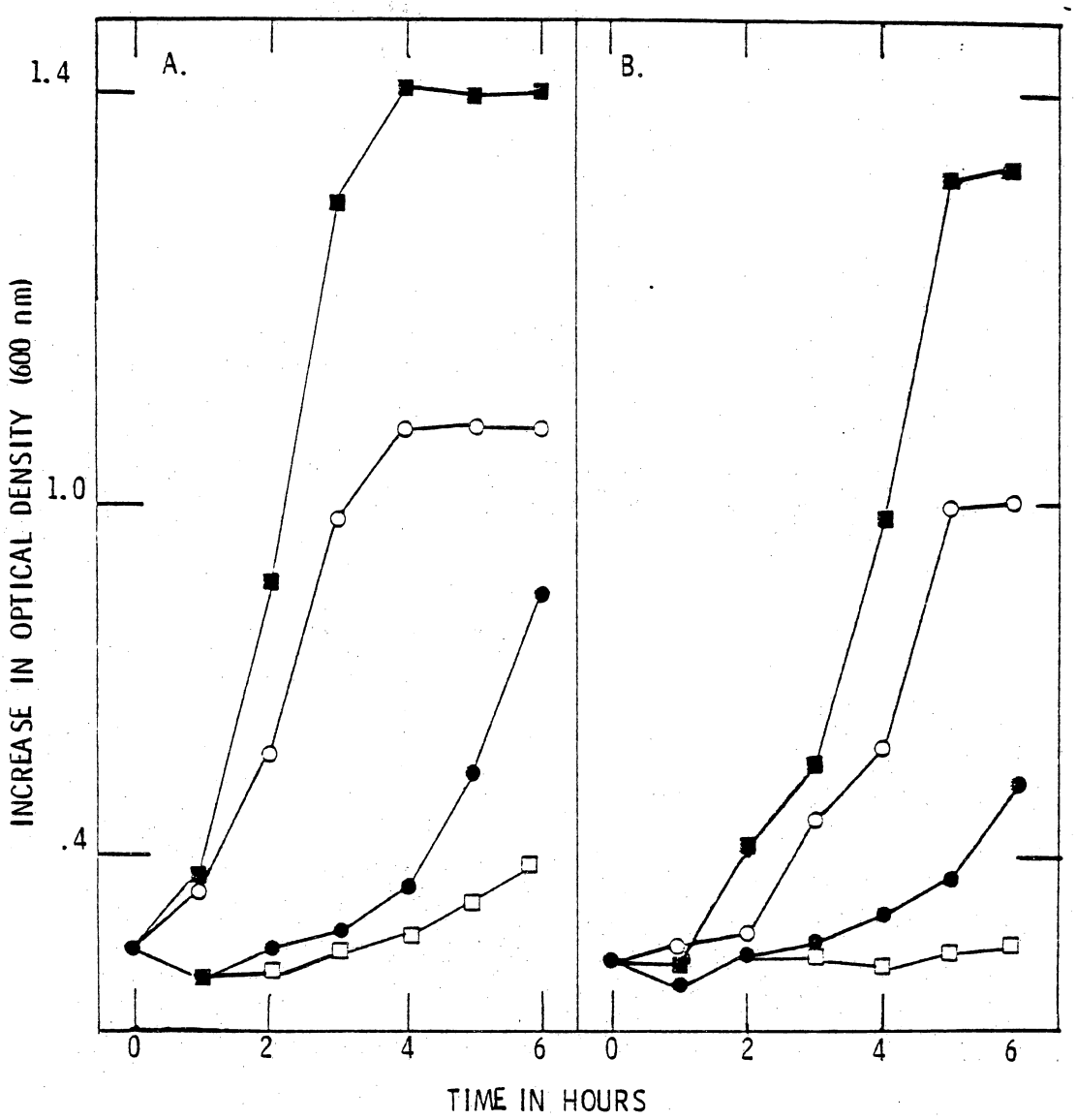

Figure 13. Effect of nitrofurantoin on growth of Lon^- and Lon^+ strains. A. Strain AB1157 (lon$^+$). B. Strain MC102 (lon-9). Cultures were incubated at 37°C with the following additions: ○, control, no additions; ■, guanosine and cytidine (100 µg/ml); ●, 5 µg nitrofurantoin per ml; guanosine and cytidine (100 µg/ml); □, nitrofurantoin (5 µg/ml).

identical results and in fact this strain was found to be as resistant to NF as AB1157. Thus, the data are in disagreement with the findings of Kirby, et al. (97) and suggest an important role for nucleotide pools in the control of cell division in Lon$^-$ strains.

6. Effect of Crystal Violet (CV) on Cell Division. The sensitivity of the Lon$^-$ strains to treatment with CV was also determined. All Lon$^-$ strains showed inhibition of division when treated with 20 μg of CV per ml in ML medium, compared to untreated control cultures (Table X and Fig. 14). In most cases, an initial increase in cell division was registered up to 30 min after exposure to the dye. This was usually following by a steady decrease in the number of survivors. Differences in susceptibility to the dye were found among all Lon$^-$ strains and a gradient similar to that found for NF resistance and UV resistance was observed. All strains tested formed filaments when exposed to CV. The lon$^+$ parent continued to divide normally and did not form filaments.

7. Ability of Isogenic Lon$^-$ Strains to Form P1 Lysogens.

The ability to form P1 lysogens is reduced in Lon$^-$ strains (165). This charactertistic can easily be measured by exposing cells to P1CMc1r100 and selecting for Cm^R transductants at 30C (70). Table XI shows the number of Cm^R transductants obtained from each isogenic Lon$^-$ strain as well as their efficiency of lysogenization by P1CMc1r100. All isogenic Lon$^-$ strains showed reduced formation of P1 lysogens since the number of Cm^R transductants obtained in each case was significantly

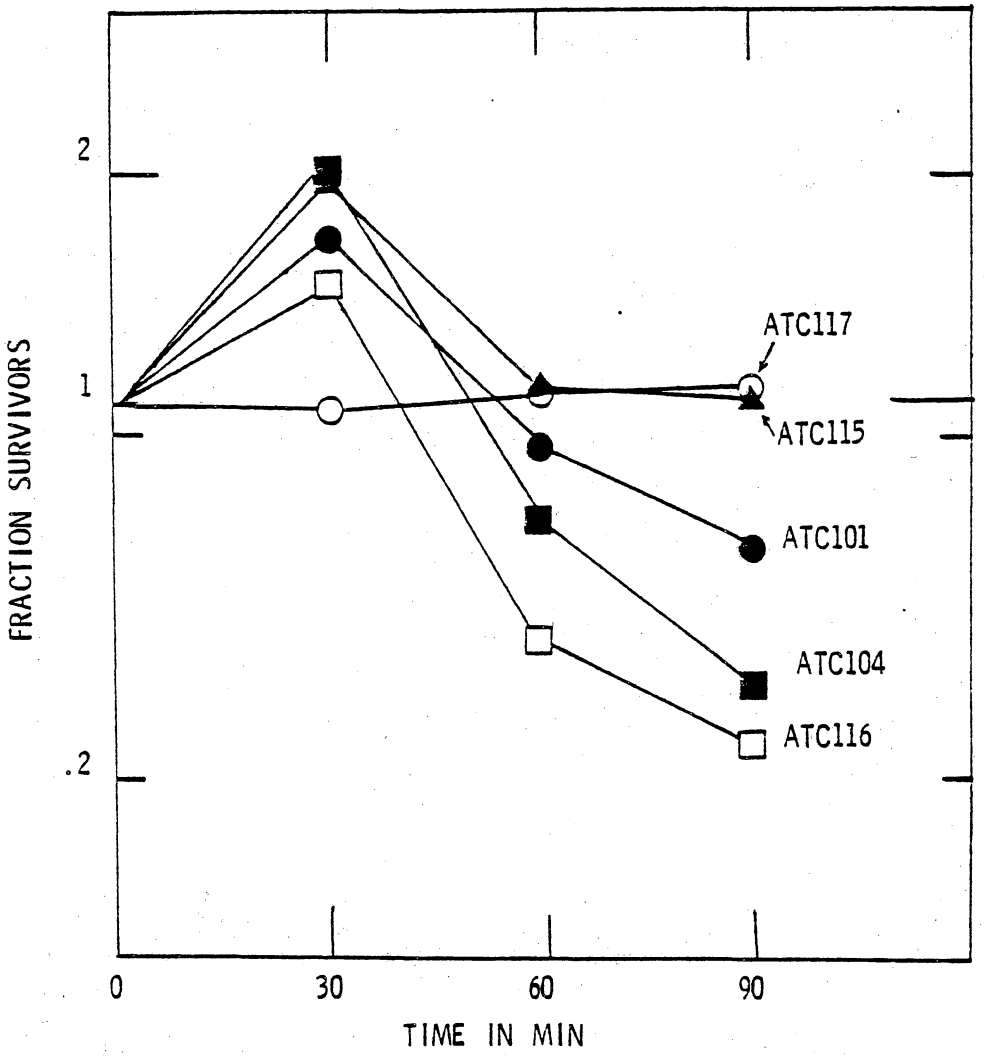

Figure 14. Effect of crystal violet on cell division of isogenic Lon$^-$ strains. Cells were grown in minimal medium to mid-log phase and crystal violet added at a final concentration of 20 µg per ml. Symbols: ○, lon-1; □, lon-7; ▲, lon-6; ●, lon-300 and ■, lon-20.

Table XI. Lysogenization by P1CM*c1r100*

Strain	*lon* allele	Cm^R Transductants per survivor	Efficiency of lysogenization by P1CM*c1r100*[a]
MC118	+	4.4×10^7	.244
ATC100	10	1×10^7	.057
ATC101	300	6×10^7	.048
ATC102	9	9×10^6	.096
ATC103	21	1.6×10^7	.052
ATC104	20	1.4×10^7	.047
ATC115	6	1×10^7	.040
ATC116	7	1×10^7	.029
ATC117	1	1.4×10^7	.037
ATC121	t-2	2.8×10^7	.065

[a] P1CM*c1r100* lysogen formation expressed as the frequency of Cm^R transductants per survivor formed at 30°C.

reduced, compared to the lon$^+$ strain. No significant differences were observed among the isogenic Lon$^-$ strains.

C. Reversion Studies of lon$^-$ Mutations.

If a mutant strain has originated as the result of a base substitution or a frameshift mutation it can be induced to revert to its original phenotype by treatment with chemical mutagens or spontaneously (3,4,35,124,158). On the other hand, if the mutation has arisen from a deletion no reversion is observed spontaneously or upon treatment with chemical mutagens (3,4,124). Thus, proof of reversion caused by a mutagen can be considered as evidence that the mutation is a point mutation or duplication. The revertants obtained include true revertants in which the original genotype has been restored, or suppressed revertants in which the original phenotype is restored because the strain carries a new, secondary mutation elsewhere in the genome that suppresses the mutant genotype (158,160).

To establish whether the lon$^-$ mutations represented in the isogenic set of strains were point mutations or deletions, the frequency of spontaneous and chemical induced reversion was measured. Base-pair substitution mutations of both the nonsense and missense type can be distinguished from both frameshift and deletion mutations since reversion varies with the type of chemical mutagen used. Base pair substitutions are readily reverted by nitrosoguanidine (NTG), but neither by 9-amino acridine (9AA) nor by the acridine half mustard, ICR-191. On the other hand, frameshift mutations can be induced to revert by 9AA and ICR-191, but not by NTG. Deletions do not revert.

Reversion analyses were done as described in section T, Materials and Methods. YET plates to which 10 μg NF per ml had been added were used to select Lon^+ revertants. The concentration of NF used was determined as described in section K, Materials and Methods. With two exceptions, high rates of spontaneous reversion were observed. Two strains, ATC101 (lon-300) and ATC103 (lon-21) failed to produce spontaneous NF^R revertants after incubation for 3 days at 37C. Strain ATC101 was reverted only by EMS and strain ATC103, was reverted by both 9AA and EMS. All other lon^- mutants were induced to revert by either NTG, EMS or both (Table XII).

The ability of certain strains (i.e., ATC100, ATC104 and ATC121) to be reverted by both NTG and ICR-191 at frequencies near or above those observed for spontaneous reversion for these strains may indicate that the MC118 genetic background could have influenced the results. When the reversion capacities of strain SG4009 (lon-t2) and the isogenic Lon^- strain derived from it (ATC121) were compared, the spontaneous and chemically induced revertant frequencies were different. Whereas spontaneous reversion of strain SG4009 was between 13 and 20 times less than chemical induced reversion, more spontaneous revertants were obtained from strain ATC121 (data not shown).

Ten separate NF^R revertants from each treatment (e.g. spontaneous and EMS-exposed) for each strain were tested further for reversion to UV resistance and non-mucoid phenotypes. All NF^R revertants tested from the isogenic strains and strain MC102 (lon-9) were UV^R, but retained

Table XII. Spontaneous and Chemical Induced Reversion of Isogenic Lon⁻ Strains[1]

Strain	lon allele	Spontaneous[a]	NTG[b]	9AA[c]	EMS[d]	ICR-191[e]
ATC100	10	+	+	-	+	+
ATC101	300	-	-	-	+	ND
ATC102	9	-	-	-	-	+
ATC103	21	-	-	+	+	ND
ATC104	20	+	+	+	-	+
ATC115	6	+	+	-	-	ND
ATC116	7	+	-	+	+	+
ATC117	1	+	-	-	+	ND
ATC120	r-1	+	-	-	+	ND
ATC121	t-2	+	+	+	+	+

[1] 10^8 cells were spread on YET plates containing 10 µg NF per ml and incubated for 72 h at 37C protected from light.

[a] Spontaneous reversion frequencies observed were between 2 and 7 x 10^{-7} revertants per ml.

[b] NTG = N-methyl-N'-Nitro-N'-nitrosoguanidine.

[c] 9AA = 9 amino acridine.

[d] EMS = ethyl methane sulfonate.

[e] ICR-191; antitumorigenic compound synthesized at the Institute for Cancer Research (Philadelphia). ND = Not determined; (+) significant numbers of revertants observed; (-) no reversion.

their mucoid phenotype; thus, all were suppressed. On the other hand, among NF^R revertants of strain SG4009, UV^R, mucoid and UV^R, non-mucoid strains were obtained. NTG was found most efficient in generating UV^R, non-mucoid revertants in this strain.

Even though the data presented here is far from conclusive, several generalizations can be made. Some lon$^-$ mutations studied could be due to point mutations, possibly to base-pair substitutions of the missense or nonsense type, since they can be reverted by alkylating agents such as NTG and EMS. The appearance of NF^R revertants that were also UV^R but retained the mucoid phenotype may indicate that these were second site revertants probably due to second site suppressor mutations similar to sul. This possibility was not tested further.

In the case of strain ATC102 (lon-9) where no revertants were obtained, spontaneously or chemically induced, the NF concentration used proved critical. When 5 μg NF per ml were used some revertants were obtained in all cases. It may well be that the selection procedure used is in itself strong enough to cause reversion in these isogenic strains. Alternatively, the results may have been affected by the nature of the chemicals used to which the Lon$^-$ strains are sensitive.

D. Detailed Characterization of UV-resistant, Mucoid (Class A) and UV-sensitive, Rough (Class B) Lon$^-$ Transductants.

The appearance of UV^R, mucoid (Class A) and UV^S, Rough (Class B) transductant classes among Pro^+ transducants of MC118 indicated that, at least, sensitivity to UV irradiation and excess production of capsular

polysaccharide could be separated in stable, non-segregating strains. It was earlier established that the degree of UV sensitivity expressed by each Lon$^-$ mutant was closely related to the position each mutation occupied at the lon locus. The intermediate classes were isolated on the basis of their degree of UV sensitivity relative to that of the lon$^+$ and lon$^-$ parental strains.

In an attempt to determine whether other Lon-associated phenotypes showed similar separation, a more detailed phenotype and genetic characterization of these strains was undertaken.

1. UV Sensitivity. To confirm that the intermediate transductant classes still showed separation of phenotypes, the degree of sensitivity to UV irradiation of each class of ProC$^+$ MC118 transductant was determined from UV survival curves (Table XIII). When the fraction of survivors of the four transductant classes after 30 sec of exposure to a UV dose of 12 ergs per sec per mm^2, is compared to that of the lon$^+$ parent, strains ATC130 (pro$^+$, lon-9), ATC132 (pro$^+$, lon-9; UV^S, Rou), ATC150 (pro$^+$, lon-1) and ATC152 (pro$^+$, lon-1: UV^S, Rou) show an increase in UV sensitivity ranging between 2 and 22-fold. On the other hand, strains ATC131 (pro$^+$, lon-9; UV^R, Muc), ATC134 (pro$^+$, lon$^+$), ATC151 (pro$^+$, lon-1; UV^R, Muc) and ATC153 (pro$^+$, lon$^+$) show between a 2 and 4-fold increase in UV resistance when compared to strain MC118. Thus, strains ATC131 and ATC151 clearly exhibit a Class A Lon phenotype while strains ATC132 and ATC152 are Class B Lon strains, based on their UV sensitivities (42). Furthermore, if their survivals after 60 sec of

Table XIII. Characteristics of Strain MC118 and its four Pro$^+$ Transductant Phenotypic Classes

Strain	lon allele	Class[a]	UV Survival[b]	Filamentation[c]	Lon Phenotype Mucoidy[d] 30C	37C	Polysaccharide[e]
MC118	+		.043	Few, mostly normal	-	-	13
ATC130	9		.0029	All, very long	+++	+	207
ATC131	9(A)	A	.098	Few, medium	+++	+	140
ATC132	9(B)	B	.0022	Few, medium to long	-	-	74
ATC134	+*		.085	Few, short, mostly normal	-	-	14
ATC150	1		.0002	All, long	+++	+	182
ATC151	1(A)	A	.1923	Many, medium	+++	-	117
ATC152	1(B)	B	.0241	Many, medium	-	-	39**
ATC153	+*		.102	Few, very short	-	-	104**

[a]Phenotypic class determined on the basis of the degree of UV sensitivity.

[b]Fraction surviving a dose of 360 ergs/mm^2 and plating on YET medium.

[c]Relative size of filaments observed in UV treated cultures 2 hr post-irradiation. Determinations were done as described in Section S, Materials and Methods.

[d]Refer to Table VII, letter c.

[e]Refer to Table VII, letter d.

(+) = mucoid; (-) = rough.

*These two strains are pro$^+$

**Only heptoses and pentoses indicated by color reaction.

exposure is compared, these differences become more marked. As shown in Figs. 15 and 16, whereas Class A strains were as resistant as the *lon*$^+$ parent, regardless of the origin of the *lon*$^-$ allele; Class B strains showed between a 7 and 450-fold increase in UV sensitivity. These differences in the degree of UV sensitivity are consistent with those found in the isogenic Lon$^-$ strains carrying either the *lon-1* or *lon-9* allele; thus, all derivatives of strain MC118 obtained with the P1(AB1899) donor were more UV resistant than MC118 derviatives obtained with the P1(MC102) donor lysate.

Regardless of the *lon*$^-$ donor used, Class A Lon transductants were more UV resistant than the Lon$^+$ parent and the ProC$^+$ Lon$^+$ transductant obtained from each cross. By contrast, strain ATC132 (Class B) was more sensitive than the Pro$^+$ Lon$^-$ transductant ATC130 and strain ATC152 was more resistant than the Pro$^+$ Lon$^+$ transductant ATC150. In addition, the Pro$^+$ Lon$^+$ transductant strains ATC134 and ATC153, showed a two-fold increase in UV resistance, compared to the *lon*$^+$ parent.

The results indicate that even though UV sensitivity was separated from mucoidy in Class A and Class B transductants, this dissociation was apparently influenced by the origin of the *lon*$^-$ donor allele. Differences in UV sensitivity were not only observed among the four classes obtained in each transductional cross but were also evident between representatives within each class.

2. *Filamentation Behavior*. The degree of filamentation observed in each transductant class is indicated in Table XIII. Two hours after

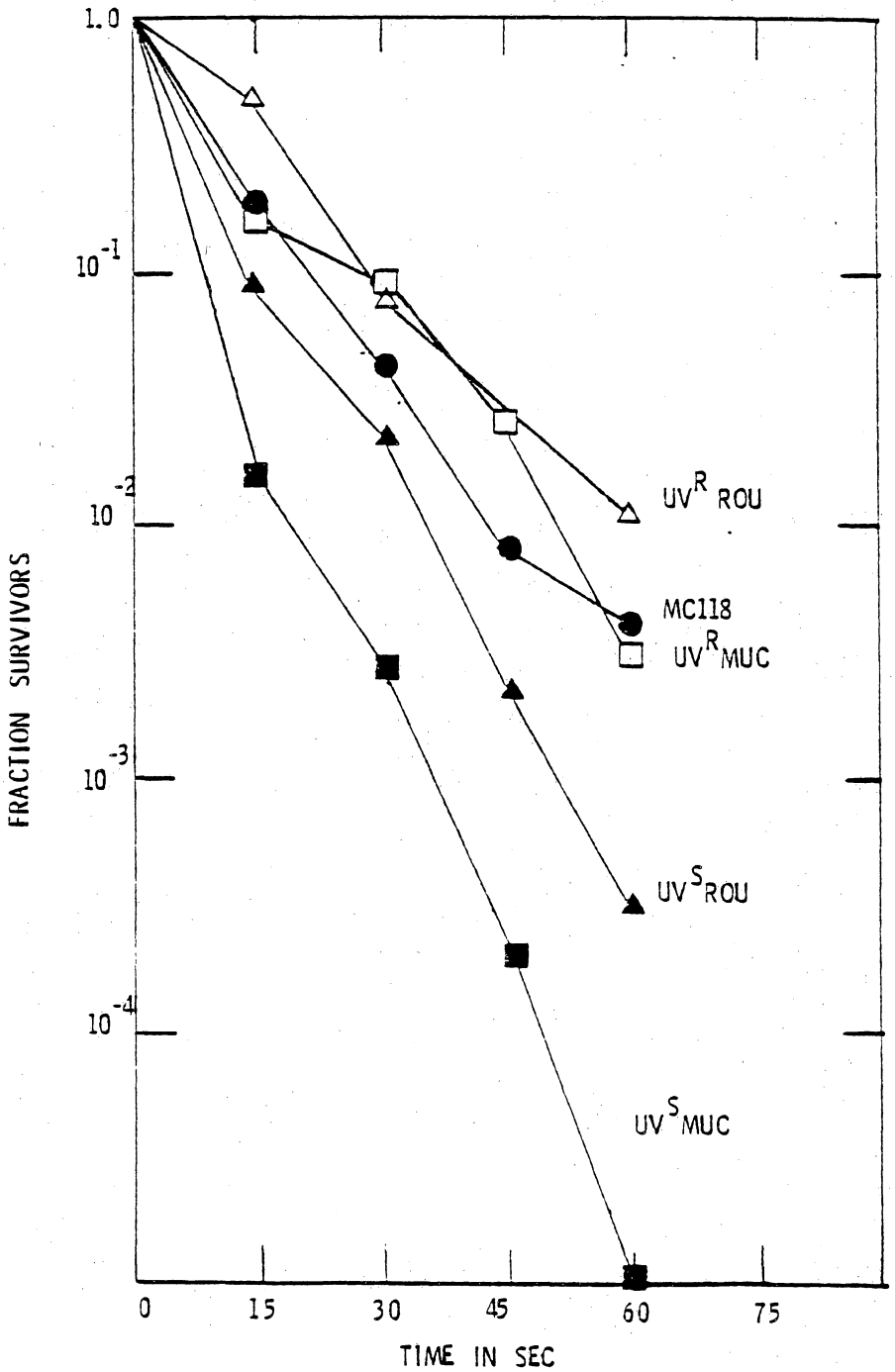

Figure 15. UV survival curves of Lon^-, Lon Class A, Lon Class B and Lon^+ Pro^+ transductants of strain MC118 obtained when P1.MC102 (proC$^+$, capR9) was used as donor. Strain designations: △, ATC134; □, ATC131; ▲, ATC132; ■, ATC130 and ●, MC118.

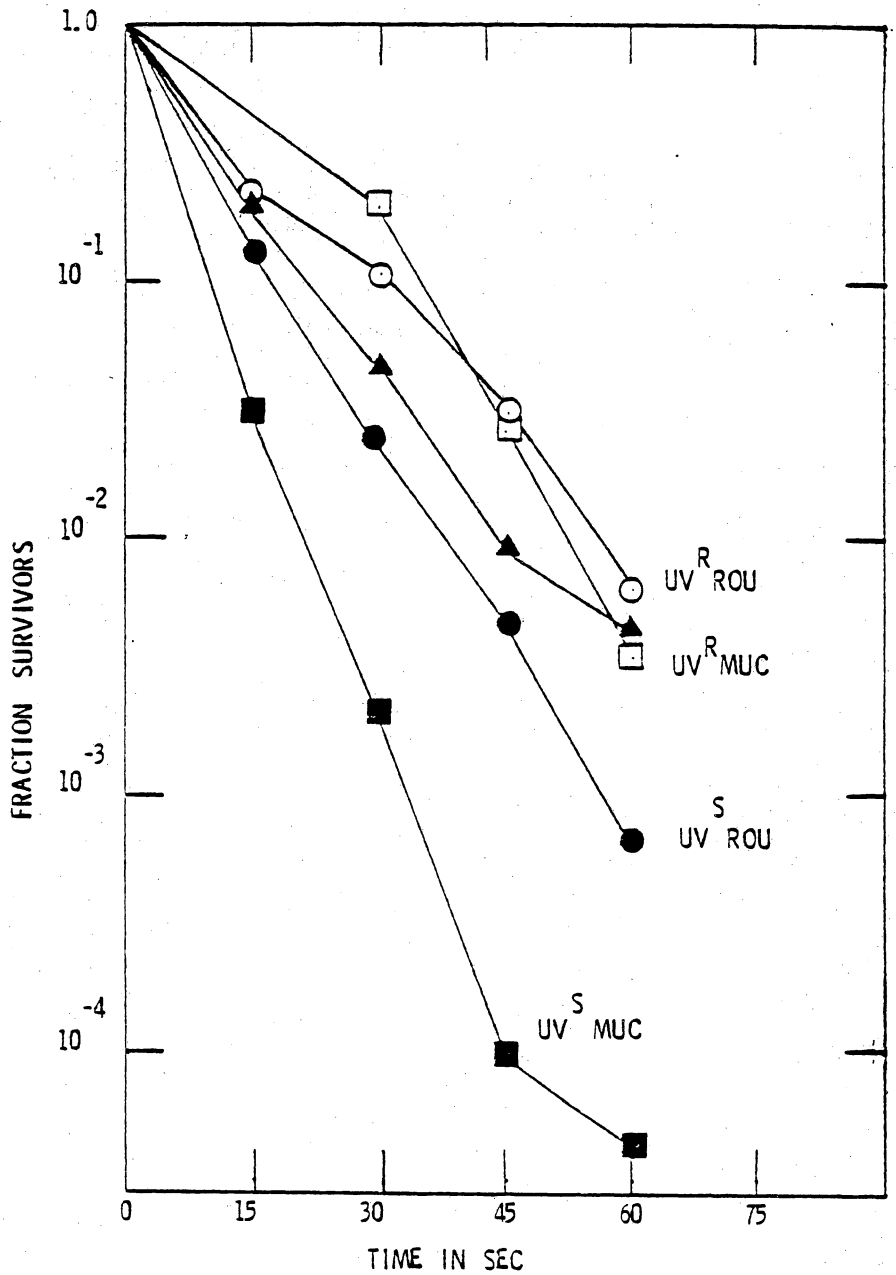

Figure 16. UV survival curves of Lon^+, Lon Class A, Lon Class B, and Lon^- Pro^+ transductants of MC118 (▲) obtained when P1.(AB1899) was used as donor. Strain designations: ○, ATC153; □, ATC151; ●, ATC152; and ■, ATC150.

exposure to UV light, Lon^- strains showed extensive filamentation. About 80% of the cells formed long filaments. Consistent with previous UV sensitivity results, strain ATC130 (UV^S, Muc; lon-9) formed longer filaments than strain ATC150 (UV^S, Muc; lon-1). The $ProC^+$ Lon^+ transductant class exhibited some degree of filamentation, but the filaments seen were short and present in small numbers.

The extent of filamentation observed for Class A and Class B Lon strains at 2 h post-irradiation, was dependent on the origin of the lon^- donor allele and intermediate between Lon^- and Lon^+ strains. Thus, whereas in cultures of strain ATC131 (lon-9A) about 30% of the cell population consisted of medium-sized filaments, about 50% of medium to long-sized filaments were observed in preparations from strain ATC132 (lon-9B). By contrast, both Class A and Class B Lon^- strains obtained from P1(AB1899) showed similar degrees of filamentation.

When examined 5 h post-irradiation, all UV^S strains showed an increase in the size and number of filaments formed. Lon^+ strains registered a reduction in the number of filaments with almost 100% normal cells. Class A strains on the other hand, showed persistence of medium-sized filaments. Therefore, even though Class A strains were more UV resistant, they were not able to overcome the transient filamentation known to occur in both Lon^+ and Lon^- strains (69). Lon^- and class B strains were unable to overcome this temporary filamentation and continued to produce long non-septate filaments even after Lon^+ strains had stopped doing so. This difference in filamentation behavior

correlates with those observed differences in UV sensitivity of these strains.

3. Production of Capsular Polysaccharide. To further establish the separation between the mucoid and UV sensitivity phenotypes, the ability to produce mucoid colonies on minimal salts medium and production of capsular polysaccharide of Class A and Class B Lon strains were determined. With one exception, all Lon^- and Lon^- Class A strains produced mucoid colonies at 30C and 37C on minimal while Lon^+ and Lon^- Class B strains gave rough, nonmucoid clones under the same conditions. Strain ATC151 (lon-1A) failed to produce mucoid colonies at 37C. The degree of mucoidy observed at 37C was much less for all mucoid strains. This was due to the presence of thymine in the medium. When thymine was eliminated all mucoid strains including ATC151 showed some degree of mucoidy at 37C, albeit to a lower extent than that observed on plates incubated at 30C.

When production of capsular polysaccharide was compared, twice as much nondialyzable methylpentose was detected in dialysates of Class A strains as compared to Class B strains. However, the data indicate that Class B (UV^S Rou) strains are still rather mucoid (e.g. strain ATC132, Table XIII) and the Class A (UV^R Muc) representative, strain ATC151, is not as mucoid as the true Lon^- strain. Thus, mucoidy in Class A and Class B strains is intermediate. By contrast, UV-sensitivity and resistance are not intermediate. Class B strains also had higher concentrations of heptoses and pentoses in dialysates than Class A strains as reflected by the specific color reactions.

4. Sensitivity to Nalidixic Acid (NA). The effect of NA on cell division of Class A and Class B Lon strains as well as Lon^+ and Lon^- $ProC^+$ MC118 transductants was determined. Cells grown to mid-log phase in either ML or YET broth were exposed to 50 μg NA per ml for 3 h. The fraction of cells surviving for each strain, after 2 h of exposure to the drug in either medium is shown in Table XIV and NA survival curves in ML for all transductant classes are presented in Figs. 17 and 18. From the data it can be seen that the response of each strain to treatment with NA depended on the lon^- donor allele and the intermediate class.

Among Pro^+ MC118 transductants obtained when P1(MC102) was used as donor, Class A Lon strains were more sensitive to NA when grown in YET broth than Class B strains. The opposite relationship was observed when the fraction of survivors of the two classes in the ML medium were compared. However, for the different classes of MC118 transductants obtained from P1(AB1899) different results were observed. All strains showed enhanced survival when treated in ML medium. Strain ATC150 ($proC^+$, lon-1) was the most nalidixic-sensitive in YET broth, whereas strains ATC153 ($proC^+$, lon^+) ws the most resistant in the same medium. When the corresponding susceptibilities to NA of these 2 strains were compared in ML medium the relationship was found to be reversed (See Table XIV). In addition, Class A strain ATC151 was more nalidixic-resistant than Class B strain ATC152 in either medium.

Table XIV. Effect of Nalidixic Acid on Cell Division of Class A and Class B Lon Strains

Strain	Genotype proC	Genotype lon	NA Survival[a] Minimal	NA Survival[a] Complex
MC118	-	+	ND	.145
ATC130	+	9	.153	.055
ATC131	+	9A	.185	.039
ATC132	+	9B	.071	.255
ATC134	+	+	.041	.19
ATC150	+	1	.394	.033
ATC151	+	1A	.337	.11
ATC152	+	1B	.297	.085
ATC153	+	+	.216	.17

[a]For survival determinations refer to Table VIII.

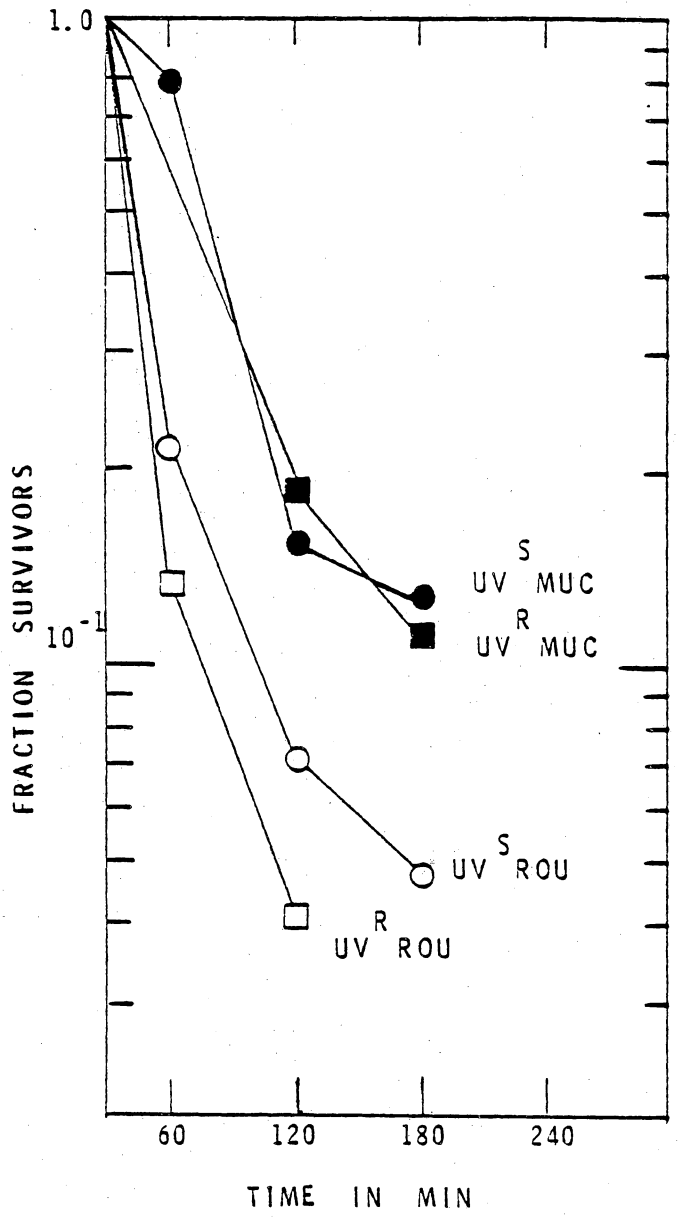

Figure 17. Effect of nalidixic acid on growth of strains ATC130, (●); ATC131, (■); ATC132, (○) and ATC134, (□). Cells were grown in minimal medium to mid-log and nalidixic acid was then added at a final concentration of 50 µg/ml.

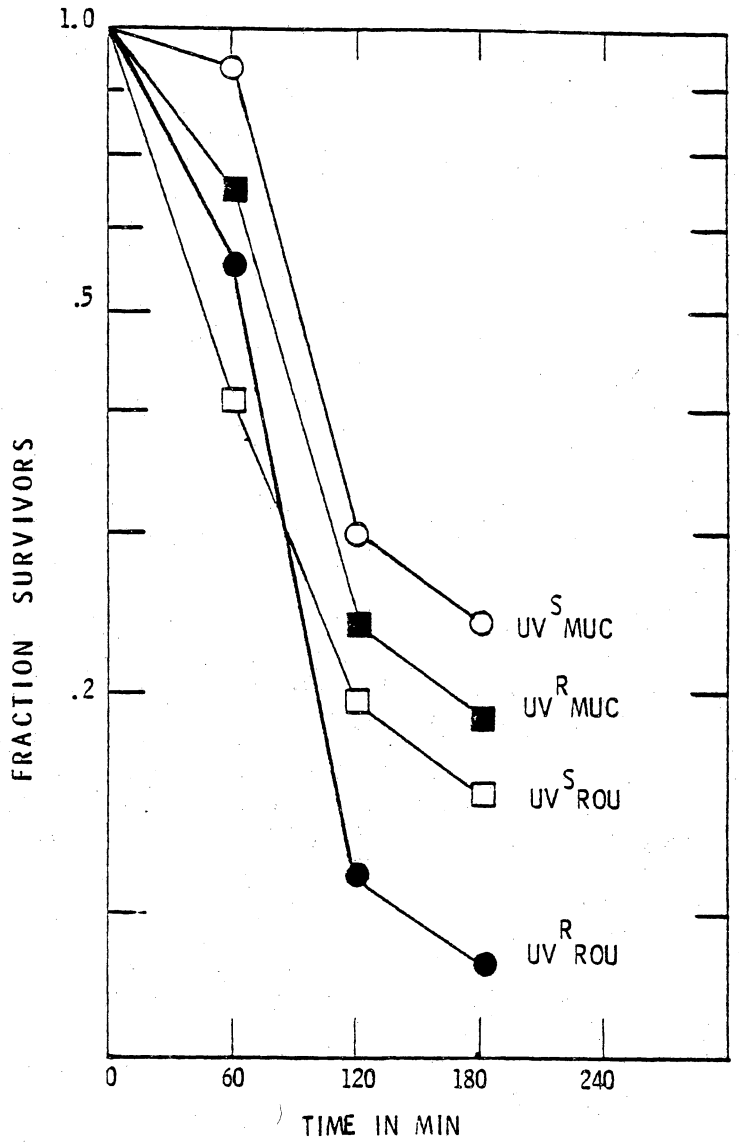

Figure 18. Effect of nalidixic acid on growth of Lon$^-$, Lon Class A, Lon Class B and Lon$^+$ Pro$^+$ transductants of strain MC118. Strain designations: ○, ATC150; ●, ATC153; □, ATC152 and ■, ATC151. For growth conditions see legend to figure 15.

As was previously seen with the isogenic Lon^- strains, the effect of NA on cell division depended not only on the genetic make-up of each strain but also on growth conditions. Growth rate of Lon^+ strains is usually faster in complex medium than that of Lon^- strains. This difference disappears whenever both Lon^+ and Lon^- cells are grown in ML medium (62). In addition, Lon^- strains, when treated with NA in minimal medium at 37C, tend to produce more capsular polysaccharide (data not shown). It is probable that enhanced growth rate of Lon^+ strains in complex medium and increased production of capsular polysaccharide by Lon^- strains in minimal medium contribute to the increased resistance to NA observed for these strains under the conditions used for these experiments. The same generalization could be derived for Class A and Class B strains obtained with P1(MC102), but not for those obtained with P1(AB1899). In the latter case, UV^R strains, regardless of their mucoid or non-mucoid phenotype were more nalidixic resistant in complex medium. However, when grown in ML medium, mucoid derivatives become more naldixic resistant. Hence, even though no definite pattern regarding sensitivity to NA was observed, the data indicates that a certain degree of separation was obtained among the four phenotypic transductant classes.

5. Crystal Violet (CV) Sensitivity. The effect of CV on cell division of Class A and Class B Lon strains was determined by growing cells to mid-log phase in ML and spreading appropriate dilutions on NA plates containing different concentrations of CV. The efficiences of

plating of all 8 transductant classes, plotted as a function of the CV concentration are shown in Fig. 19. As expected, Lon^- strains were CV^S while Lon^+ strains were CV^R. Based on their sensitivities to CV, the strains tested could be classified in one of three classes. Strains ATC132 (lon-9B), ATC134 (lon^+) and ATC153 (lon^+), all non-mucoid, were resistant to CV concentrations higher than 30 µg per ml and are considered to be CV^R. The second group of strains was able to grow at CV concentrations between 10 and 30 µg per ml and included strains ATC152 (lon-1B) and ATC131 (lon-9A). The third group, composed of strains ATC130 (lon-9), ATC150 (lon-1) and ATC151 (lon-1A), showed no growth at a CV concentration of 10 µg per ml. These results showed that Class B strains were more CV^R than Class A strains, a pattern similar to that seen for NA sensitivity.

6. Effect of Nitrofurantoin (NF) on Cell Division and Growth of Class A and Class B Lon Strains. The effect of NF on cell division of Class A and Class B Lon transductants was determined as described in Section D, Materials and Methods. Fig. 20 shows the results obtained with strains ATC130 (lon-9), ATC131 (lon-9A), ATC132 (lon-9B) and ATC134 (lon^+). For all four strains, a marked decrease in survival was observed after 90 min of continuous exposure to 5 µg of NF per ml in minimal medium. The largest decrease was seen for strains ATC130 (Lon^-) and ATC131 (Class A) since these two strains showed a 13-fold increase in sensitivity to NF as compared to the lon^+ parent. Class B strain ATC132 showed a 7-fold decrease in survival while the $proC^+$ lon^+

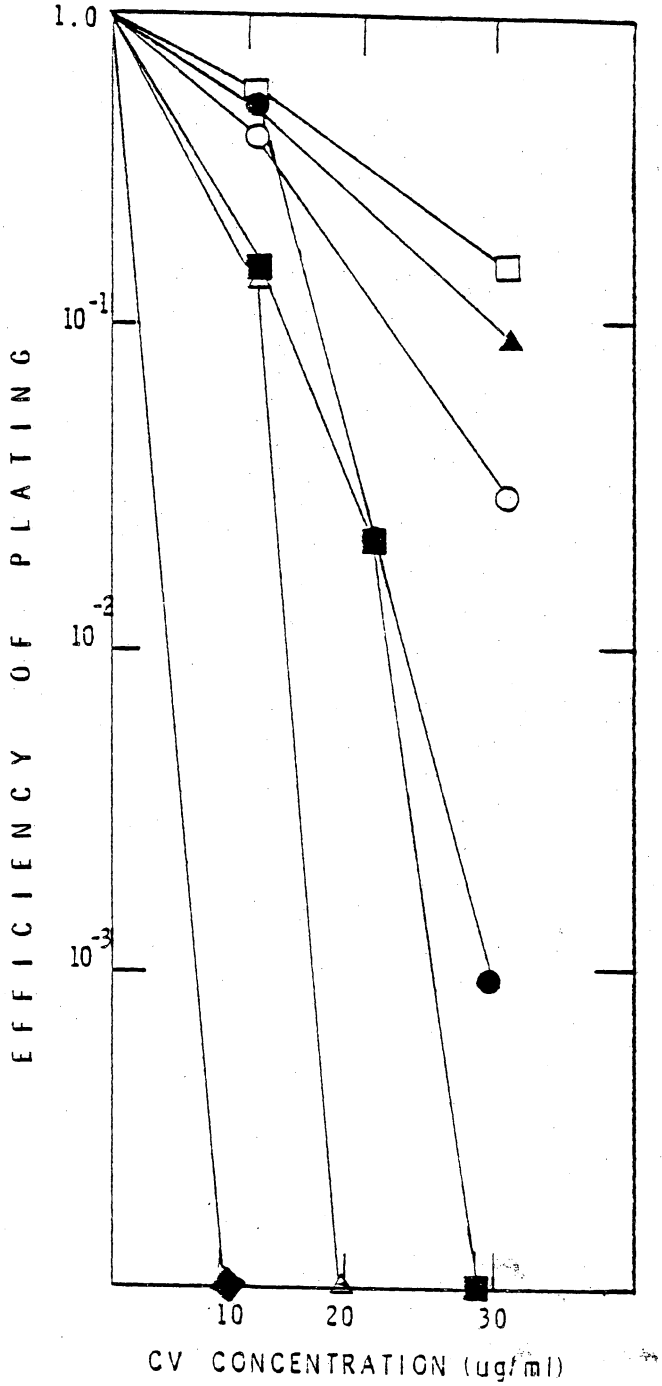

Figure 19. Efficiency of plating of Class A and Class B Lon strains on minimal medium containing crystal violet. The efficiency of plating is plotted against CV concentration. Symbols: □, ATC132 (lon-9B); ○, ATC134 (lon$^+$); △, ATC131 (lon-9A); ●, ATC153 (lon$^+$); ■, ATC152 (lon-1B); ♦, ATC151 (lon-1A), ATC150 (lon-1), ATC130 (lon-9); ▲, MC118 (lon$^+$).

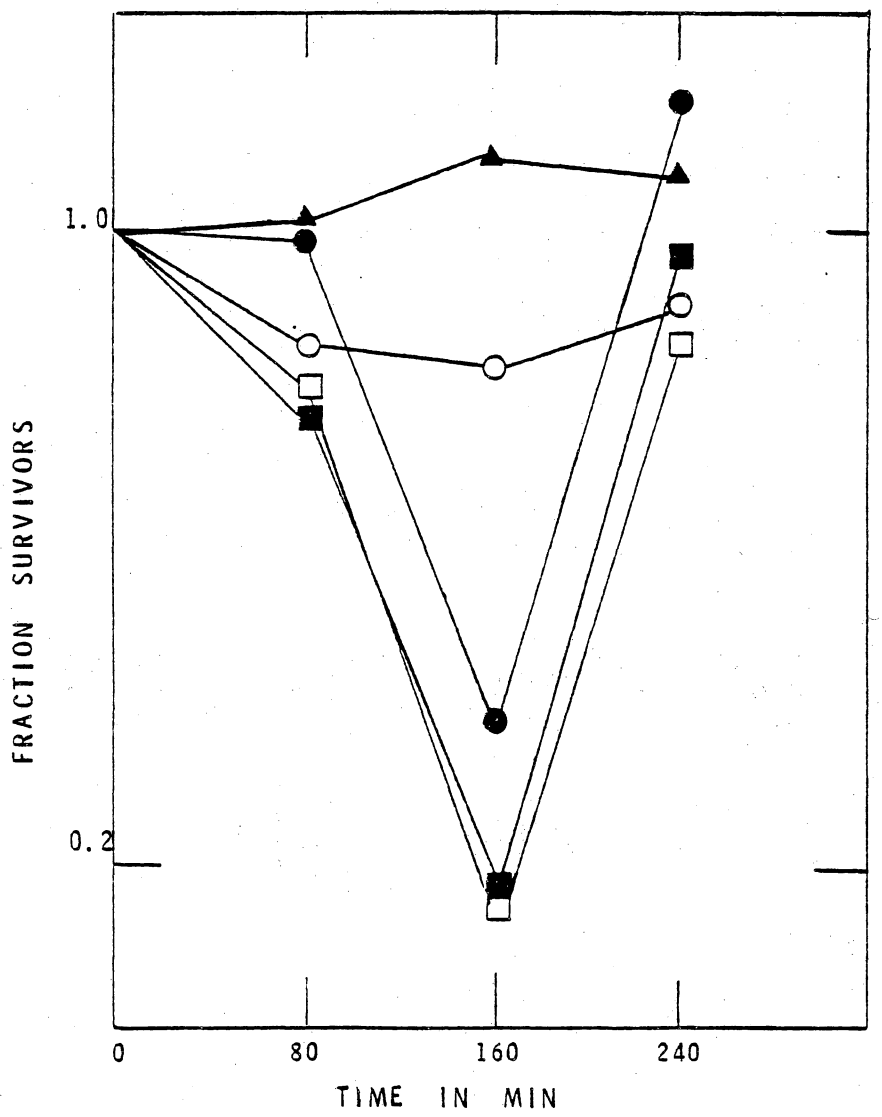

Figure 20. Effect of nitrofurantoin on cell division of strains ATC130 (●), ATC131 (□), ATC132 (■) and ATC134 (○). Cells were grown in minimal medium to mid-log phase. NF was added at a final concentration of 5 µg/ml.

transductant strain was about twice as NF^S as strain MC118 (lon$^+$). After 3 h of continuous exposure cell division was resumed in all strains, with strains ATC130 and ATC132 showing the largest increase in survival. On the other hand, strain ATC134 (proC$^+$, lon$^+$) failed to show this increase and continued to divide at a slower rate than that observed for strain MC118.

The results obtained with strains ATC150 (lon-1), ATC151 (lon-1A), ATC152 (lon-1B) and ATC153 (proC$^+$, lon$^+$) are shown in Fig. 21. All strains were more NF resistant than those strains derived from lon-9. The survival curves indicate that after 180 min of continuous exposure to 5 μg of NF per ml in minimal medium, strains ATC150 (Lon$^-$) and ATC151 (Class A) showed the most marked decrease in survival. Strain ATC152 (Class B) showed an initial 90 min lag in cell division after which the division rate increased slowly. On the contrary, an initial decrease in survival was observed for strain ATC153 during the first 90 min of exposure after which a steady increase in cell division was observed. After 3 h exposure, whereas strain ATC150 (Lon$^-$) was able to completely overcome the division block, strain ATC151 (lon-1A) continued to show a steady decrease in survival. As seen before, the Pro$^+$, Lon$^+$ transductant had a lower rate of cell division when compared to the Pro$^-$ Lon$^+$ parent (MC118).

When UV-irradiated, filamenting Lon$^-$ cells growing in complex medium are shifted to minimal medium the cells return to their normal morphology. The increase in cell division observed in most of these

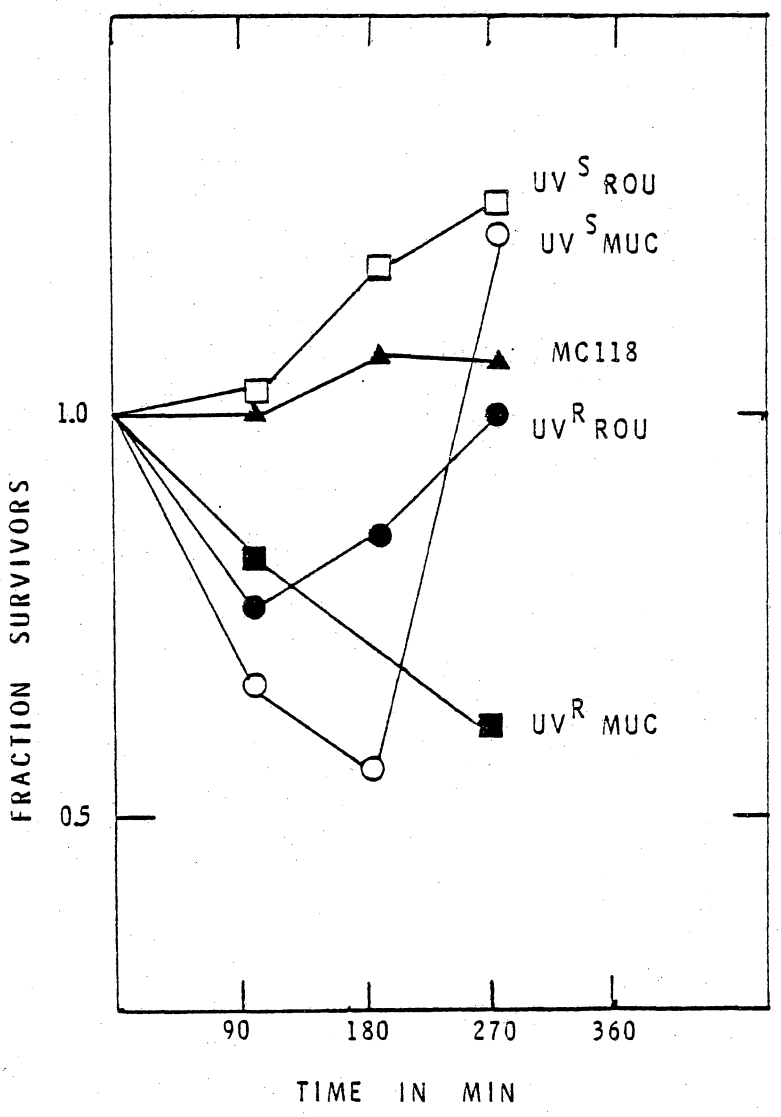

Figure 21. Effect of nitrofurantoin on cell division of strains ATC150 (○), ATC151 (■), ATC152 (□) and ATC153 (●). Cells were grown in minimal medium containing 5 µg NF per ml.

strains after 3 h of continuous exposure to NF could then be attributed to the reversion of the NF-induced filamentation that occurs when the cells are incubated with the drug in minimal medium. In fact, with one exception, after 4.5 h of exposure to NF, filaments were no longer present in the treated cultures. The cell division inhibition persisted in strain ATC151 (Class B) after the same time period. It cannot be deduced from the data whether this was due to a permanent block in cell division or to a longer lag period preceding recovery in this strain.

If the effect of NF on cell division of Class A and Class B strains grown in minimal medium is compared, Class B derivatives are sensitive to nalidixic acid but nitrofurantoin resistant, while the opposite relationship holds for Class A clones. When sensitivity to nalidixic acid in complex medium of Class A and Class B strains is compared to NF sensitivity, the same relationship is observed for intermediate classes obtained from the lon-1 donor, whereas Class A strain ATC131 (lon-9A) shows increased NA^S (higher NF^S) and Class B strain ATC132 (lon-9B) shows increased NA^R (higher NF^R).

7. Effect of NF and Nucleosides on Growth of Class A and Class B Lon Strains. The effect of NF and of guanosine and cytidine on growth of Class A and Class B Lon strains was determined. Figs. 22 and 23 show the results obtained. Addition of guanosine and cytidine (100 μg/ml) to cultures treated with 5 μg NF per ml in minimal medium, enhanced cell division of Class B strains. The treatment was less effective with Class A strains. In the absence of NF, guanosine and cytidine greatly

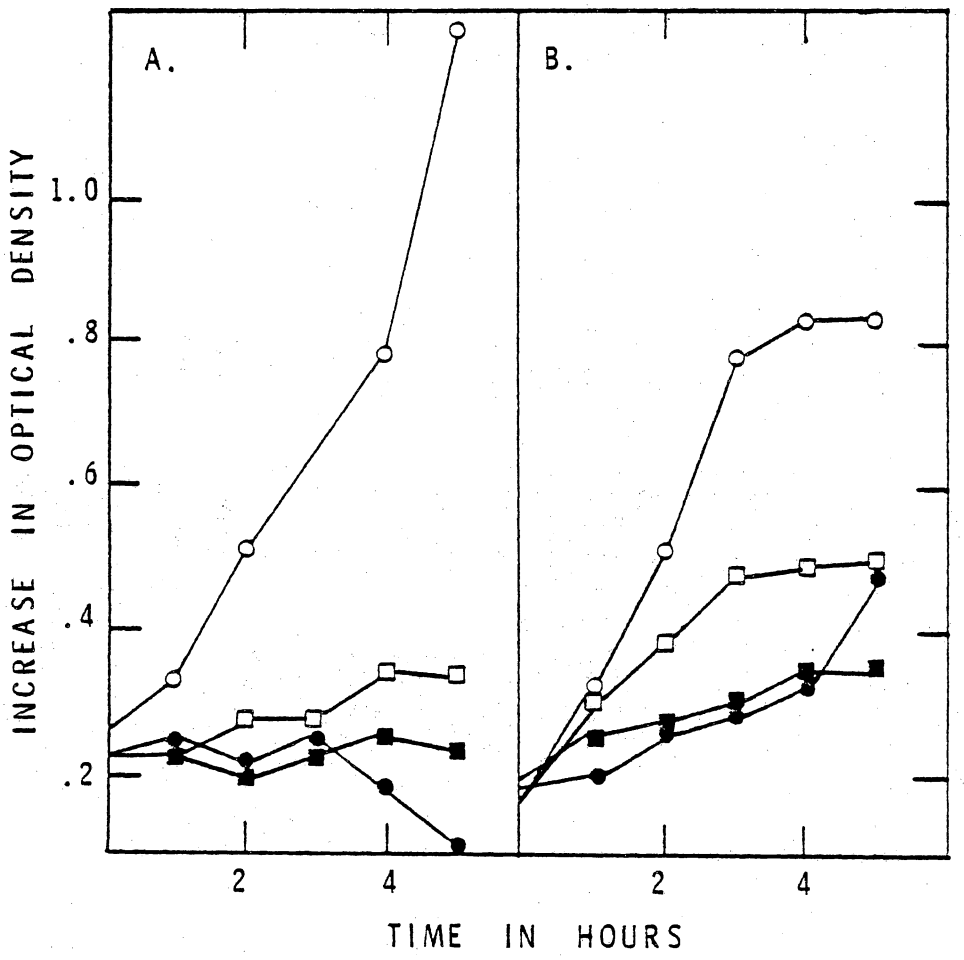

Figure 22. Effect of nucleosides on growth of Class A and Class B Lon strains. Figure 21A: ATC151 (lon-1A); Figure 21B: ATC152 (lon-1B). Cultures were incubated at 37C with the following additions: ●, control, no additions; ○, guanosine and cytidine (100 µg/ml); ■, 5 µg nitrofurantoin per ml; □, 5 µg nitrofurantoin per ml and guanosine and cytidine (100 µg/ml). Optical density was measured at 600 nm.

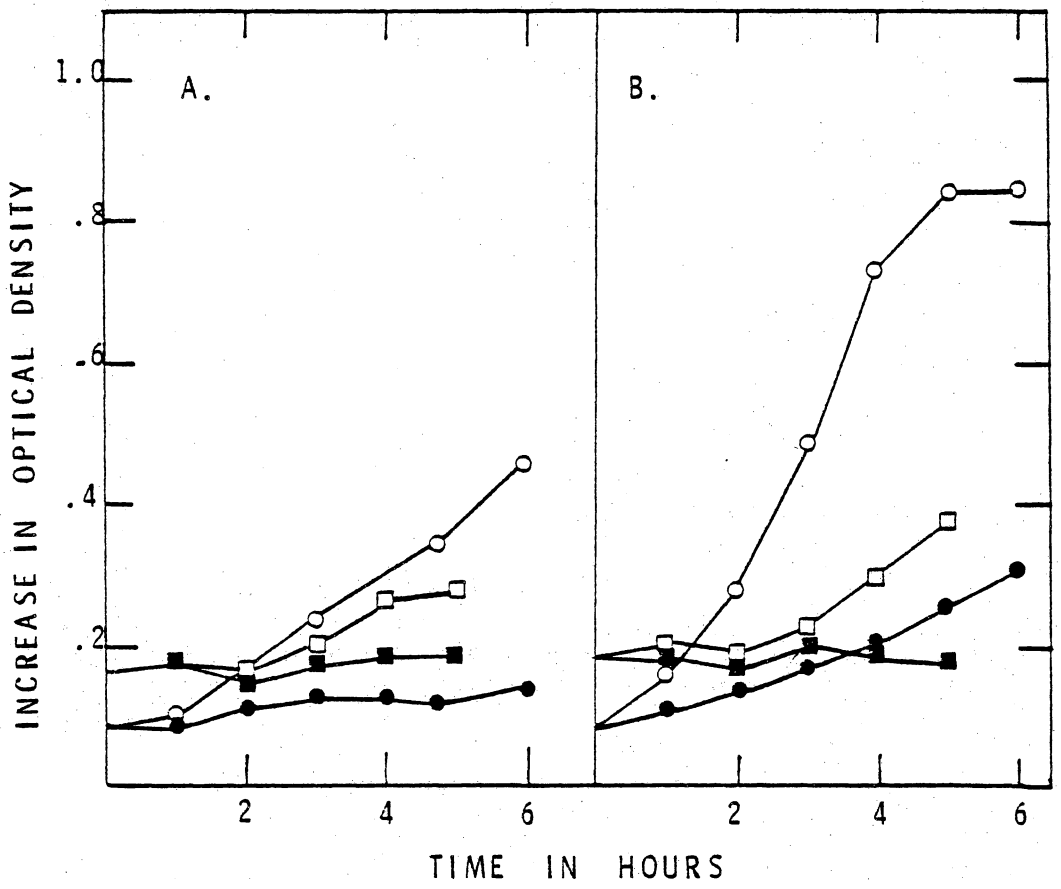

Figure 23. Effect of nucleosides on growth of Class A and Class B Lon strains. Figure 21A: ATC131 (lon-9A). Figure 21B: ATC132 (lon-9B). For culture conditions refer to legend of Figure 21.

enhanced cell division of Class B strain ATC132. On the other hand, a less pronounced effect was seen when the same nucleosides were added to Class A strain ATC131. The reverse relationship was found for strains ATC151 and ATC152.

8. P1CMc1r100 Lysogen Formation. The frequency of formation of P1CMc1r100 lysogens by Class A and Class B Lon strains as well as that of strains MC118 (lon$^+$) MC102 (lon-9) and AB1899 (lon-1) are shown in Table XV. Among all strains derived from MC118, only ATC130 (lon-9) showed enhanced ability to form P1CMc1r100 lysogens compared to the Lon$^-$ donor strain MC102. On the other hand, strain ATC150 (lon-1), exhibited a slight decrease in lysogen formation, compared to the Lon$^-$ donor AB1899.

The extent to which P1CMc1r100 lysogens were formed by Class A and Class B Lon strains was also dependent on the lon$^-$ donor allele. Whereas representatives of both intermediate classes of Lon derivatives obtained with the P1(MC102) donor showed a decrease in the ability to form P1CMc1r100 lysogens compared to strain MC118, the respective Lon$^-$ intermediate derivatives obtained with the P1.AB1899 donor were more capable of forming lysogens. Nevertheless, when comparisons are made between them, Class A (UV^R Muc) strains are found to be more proficient in forming P1 lysogens than Class B (UV^S, Rou) strains, suggesting a role for capsular polysaccharide in the adsorption of phage.

Contrary to what was expected, the Pro$^+$ Lon$^+$ transductant strains showed a 2-4 fold decrease in P1 lysogen formation compared to that of

Table XV. P1CM*c1r100* Lysogenization of MC118 Lon Transductants

Strain[a]	*lon*-allele	Frequency of P1 lysogens[b]
MC118	+	.0175
ATC130	9	.0115
ATC131	9A	.0065
ATC132	9B	.0048
ATC134	+	.0035
ATC150	1	.0091
ATC151	1A	.0260
ATC152	1B	.0150
ATC153	+	.0092

[a]Strain phenotypes as in Tables XIII and XIV.

[b]Refer to Table XI.

the *lon*$^+$ parent. Furthermore, strain ATC134 (Lon$^+$) was about 3 times less able to form P1 lysogens than strain ATC130 (Lon$^-$). These, results suggest that the differential expression of this phenotype could be dependent on the position of each allele at the *lon* locus, or to an MC118 mutation, unlinked to *proC*.

9. *Burst Size of Temperature-Sensitive λ*. To establish whether the ability to stabilize missense polypeptides was affected in Class A and Class B Lon transductants and thus, indirectly measure the Deg$^-$ phenotype in these strains, the burst size of the temperature sensitive phage λcI857 (Ot^s) was determined. No significant differences were obtained (Table XVI) among Lon$^-$, Lon (Class A) and Lon (Class B) transductants of P1(MC102) (*lon-9*). Class A and Class B Lon strains derived from P1.AB1899 instead, showed between a 1.6 to 2-fold increase in burst size when compared to the Lon$^-$ transductant and about a 5-fold increase above that observed for strain MC118. Thus, the presence of the *lon-1* allele leads to an enhancement of the stabilization of missense polypeptides above that observed in strains carrying the *lon-9* allele. This response is similar to that observed whenever P1C*Mc1r100* lysogen formation was tested in these strains.

10. *Analysis for the Presence of Known Suppressors of lon: sulA and sulB*. The UV^R, mucoid phenotype of Class A transductants could be due to the presence of known suppresors of *lon*$^-$, namely *sulA* (*pyrD* linked) or *sulB* (*leu* linked), which abolish UV sensitivity and filamentation (69,89). Alternatively, the presence of a *non* mutation in

Table XVI. Burst Size of Temperature Sensitive λ.

Strain[a]	Genotype pro	Genotype lon	λcI857(Ot^s)[b] Burst Size
MC118	-	+	1.7
ATC130	+	9	18.9
ATC131	+	9A	19.8
ATC132	+	9B	12.8
ATC134	+	+	9.7
ATC150	+	1	27.5
ATC151	+	1A	45.0
ATC152	+	1B	57.1
ATC153	+	+	7.3

[a]Refer to Table IV.

[b]Burst size of temperature-sensitive λ phage normalized against burst size of parental phage, was determined as described in Section 2, Materials and Methods.

the MC118 genetic background could be responsible for the appearance of UV^S, Rou, Class B transductants, although this possibility seems unlikely.

To investigate the possibility of any sulA or sulB mutation being present in the recipient, P1CMc1r100 was grown on a leu^+ derivative of strain MC118 (ATC802) and each one of the Class A transductants ATC131 and ATC151. The resulting lysates were then used to transduce strain SG13022 (leu, pyrD, lon- 100) with subsequent selection for either Leu^+ or Ura^+ transductants, which were then tested for expression of the mucoid phenotype and resistance to UV and MMS. All Ura^+ clones tested were mucoid, UV^S and MMS^S; thus, a sulA mutation was not present in these strains. Likewise, all Leu^+ clones tested were still mucoid, UV^S and MMS^S, indicating that no sulB mutation existed in the MC118 background. Therefore, the appearance of UV^R, mucoid colonies was not due to the presence of known outside suppressors of lon^-.

11. Genetic Analysis of Class A and Class B Lon Strains. If the two Lon phenotypes, mucoidy and UV sensitivity had actually been separated in Class A and Class B Lon strains, then it could be expected that in crosses between P1 grown on these strains and strain MC118 (a backcross) the phenotypes should breed true. This possibility was tested further with strains ATC131 (lon-9A) and ATC132 (lon-9B). P1CMc1r100 grown on each of these two strains was used to infect the parental strain MC118 ($proC^-$, lon^+) with subsequent selection for Pro^+ transductants.

All Pro^+ transductants tested from the Class B (UV^S Rou) donor retained that same phenotype. However, among the Pro^+ transductants with the Class A donor (UV^R Muc), 4% that appeared UV^R Muc in the primary selection proved to be UV^S, Muc upon secondary screening (Table XVII). Thus, the separation of UV sensitivity and mucoidy in transductants may not be due to actual genetic separation of two distinct genes. Perhaps complete separation of phenotypes cannot be achieved and only variations in the degree of expression of mucoidy and UV sensitivity (as well as of other Lon-associated phenotypes) can be obtained. These gradients could be determined by the "clusters" of mutations at this locus, which could influence not the expression of one or more genes but rather the expression of a specific function or set of functions determined by the lon locus. That is, the lon mutations studied represent two domains of a single gene whose product is a multifunctional polypeptide.

E. Complementation Studies. The results of the genetic analysis of Class A and Class B Lon^- strains (Section 11, Results) suggested that the lon mutations studied could represent two domains of a single gene having a multifunctional polypeptide as its product, thus ruling out the presence of two genes at this locus. These two conditions can be easily distinguished from each other in complementation studies.

If the lon locus consists of two discrete functional units, complementation would be expected to occur only between "clusters" of alleles but not within them. On the other hand, if the locus encodes

Table XVII. Testing Class A and Class B Strains by Back transduction[1]

lon allele	Donor Phenotype UV	Muc	Transductants: ProC+ Mucoid Primary Selection % Muc	UVS screening % UVR	% UVS	ProC+ Nonmucoid Primary Selection % Rou	UVS screening % UVR	% UVS
lon-9	R	+	9.8	96.0	4.0	90.2	100	0
	S	-	0	0	0	100	60.6	39.4

[1]Recipient was strain MC118. For genotype refer to Table II.

for a single polypeptide (or functional unit) there could be either no complementation between alleles or intragenic complementation (55) between *lon*$^-$ mutations located possibly in different functional domains.

To investigate these two possibilities the complementation behavior of all 10 *lon*$^-$ alleles was analyzed. An isogenic set of *recA*$^-$ strains was constructed from the isogenic set of Lon$^-$ strains derived from strain MC118, by infecting them with P1 (χA21) which carries a Tn10 insertion in the *recA* gene, and selecting for Tc^R transductants (98,152). These strains were then used as recipients in plate matings in which the isogenic F'-*lon*$^-$ strains were the donors. Because Lon$^-$ strains are sensitive to NF and MMS, either one of these two agents was used in the selective medium. Significant differences in sensitivity to these agents were found between F' *lon*$^-$ donors and F$^-$ *lon*$^-$ recipients. F' *lon*$^-$ donors were more resistant to higher concentrations of both agents than the recipients, probably due to different genetic backgrounds. Furthermore, selection was hindered by the inherent extreme sensitivity of *recA*$^-$ strains to UV or any other radiomimetic agent (such as MMS). Therefore, NF at a final concentration of 5 μg/ml was added to LB agar plates containing 100 μg Sm per ml (See Section V, Materials and Methods) and used as selective medium. Selection was made for NF^R colonies.

The complementation behavior of *lon*$^-$ alleles is shown in Table XVIII. Four distinct patterns emerged from the analysis of the data. Five alleles (*lon-1*, *lon-6*, *lon-7*, *lon-9*, *lon-21*) showed positive

Table XVIII. Complementation Behavior of Lon$^-$ Alleles[a]

F$^-$Recipients	F'Donors 10	300	r1	t2	6	1	21	9	7	20
7	1	1	1	1	+	+	+	+	2	-
9	1	1	1	1	1	+	+	2	+	-
21	-	1	1	1	1	-	2	+	+	-
6	-	1	1	1	-	1	-	-	+	-
1	-	-	-	-	-	-	-	+	+	-
r1	1	-	-	1	1	-	-	-	-	-
20	-	-	-	-	1	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
300	-	-	-	-	-	-	-	-	-	-
t2	-	-	-	-	-	-	-	-	-	-

[a]F'donors carrying the *lon*$^-$ alleles on the plasmid were crossed to F$^-$ *lon*$^-$ *recA*$^-$ recipients as described in Materials and Methods, Section V.1. Selection was done on LB plates containing 5 µg NF per ml and 100 µg streptomycin sulfate per ml. Matings were incubated at 37C for 72 h protected from light. Symbols: (+) = heavy patch growth; (-) = no growth; (1) = crosses with few colonies, possibly revertants or recombinants. Reciprocal crosses showed no growth; (2) = selfing crosses where a few colonies were seen possibly revertants. The ordering of alleles is arbitrary.

complementation in certain combinations regardless of the direction of the cross. Other combinations of alleles (represented by 1 on Table XVIII) produced isolated colonies in one direction, but reciprocal crosses showed no growth. The appearance of colonies in these crosses could be due to reversion, to production of rare recombinants, to leakiness of the mutants involved, or to the presence of an "unlinked" suppressor gene.

A third pattern of complementation was observed among the alleles lon-r1, lon-20, lon-10, lon-300, and lon t-2. These alleles failed to complement each other in all combinations tested as well as with all other remaining alleles. In addition, apparent "selfing" occurred in crosses involving the lon-21, lon-9 and lon-7 alleles (indicated by a number 2 in Table XVIII), but these produced few, isolated colonies; probably the result of reversion.

When those combinations of alleles which registered positive complementation are considered, and a complementation map based on the data in Table XVIII is compared with the linkage map obtained previously (Fig. 24), two observations can be made: (1) the genetic and complementation maps are not separated into discrete, distinct segments; (2) noncomplementary mutations are found in segments which overlap those in which complementing mutations are found.

These results strongly suggest that the lon gene produces a single polypeptide which is able to perform several distinct, independent functions (domains) with mutations along the length of the gene causing

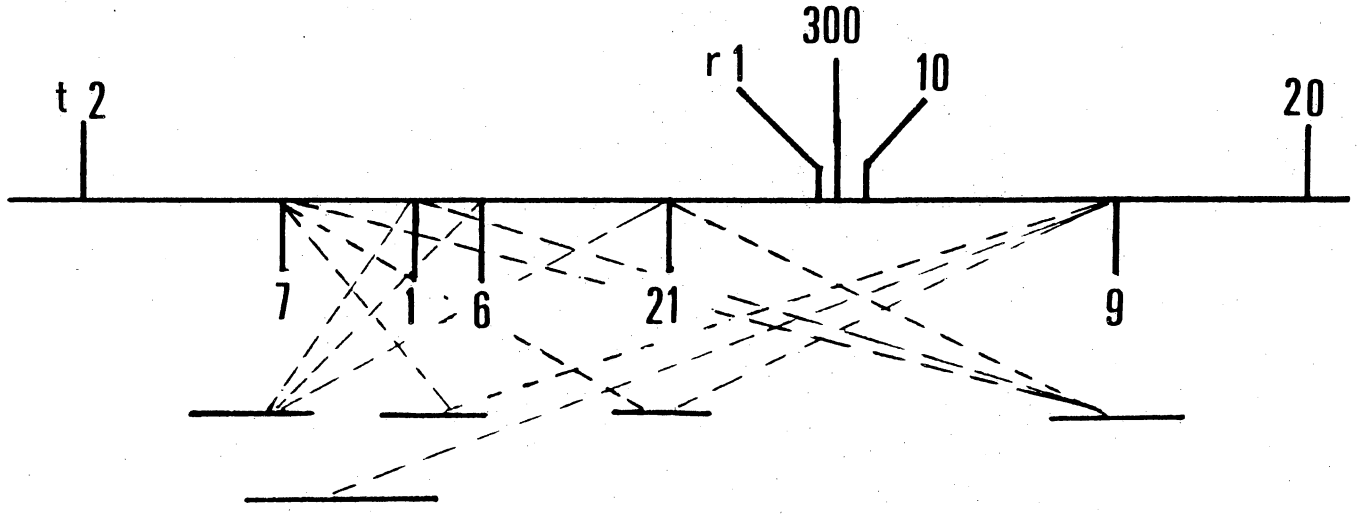

Figure 24. Complementation map of the lon locus compared with the genetic map. Mutants above the line do not complement each other or any other member of the series. Complementing alleles are shown connected by broken lines to the corresponding segment on the complementation map. Alleles lon-10, lon-300 and lon-r1 probably represent the same mutation.

differential expression of these functions. This is probably mediated by changes in the quaternary structure of the tetramer (27,28) through modifications in folding patterns of the molecule in much the same way as has been observed in the case of the homomultimeric enzyme alkaline phosphatase in Escherichia coli (55).

F. Recombination Studies. To determine whether genetic recombination could occur between representative lon$^-$ alleles from each one of the two "clusters" of mutations found at the lon locus, spot matings between strains carrying the lon-1 and lon-9 alleles were done. These alleles were selected because they had previously shown clear cut differences in the expression of the Lon-associated phenotypes tested.

An isogenic set of F' strains was constructed in which the lon$^-$ alleles were placed on the plasmid by infecting strain ATC125, a proC$^-$ derivative of strain χ573 (a primary F-prime carrying the lac to minE region on the plasmid) with P1CMc1r100 lysates of strains MC102 and AB1899, and subsequent selection for Pro$^+$ mucoid transductants. Strains ATC302 (F' lon-9) and ATC317 (F' lon-1) were then crossed with the F$^-$ strains ATC130 (lon-9) and ATC150 (lon-1) in all possible combinations to investigate possible differences that could be due to either episomal dominance, the direction of the cross or both. Crosses between an F' lon$^+$ donor (ATC125) and strains ATC130 and ATC150 were also done. Spot matings and subsequent characterization of the resulting merodiploid strains were done as described in Section V, Materials and Methods.

The results of these experiments are shown in Table XIX. Possible Pro^+ recombinants were selected from spots in which 25 or more isolated colonies had been observed. Recombination between the lon-1 and lon-9 alleles resulting in UV^R Rou NF^R colonies occurred, regardless of the direction of the cross, although the frequencies obtained cannot be considered as reliable indicators of the distance between the two mutations since a reduced number of colonies were screened. By contrast, selfing crosses (ATC302 x ATC130; ATC317 x ATC150) failed to produce lon^+ (UV^R, Rou) recombinants.

In crosses where the lon^+ allele was on the plasmid, half of the colonies screened were UV^R, Rou (Lon^+). However, in reciprocal crosses where the lon^+ allele was on the chromosome and the lon^- alleles on the plasmid only 10% of the colonies screened were UV^R although mucoid. Furthermore, some intermediate UV^R Muc (Class A) and UV^S Rou (Class B) recombinants were observed in certain crosses (ATC302 x ATC150; ATC317 x ATC130; ATC125 x ATC130). Even though the data shown is not conclusive, the appearance of UV ^{R} Rou (Lon^+) merodiploids that were $MS2^R$ indicates that recombination between the lon-1 and lon-9 alleles did occur.

G. Isolation of Deletions

Attempts to generate deletions of the lon gene of the plasmid of strain χ573 by nitrous acid mutagenesis proved difficult. Treatment of cultures with the mutagen and selection were done as indicated in Section U, Materials and Methods. Fifty mucoid $T6^R$ colonies from each one of fifteen independently treated cultures were screened for mucoidy

Table XIX. Recombination Between *lon-1* and *lon-9* alleles[a]

Cross			F'-ductants			
Donor	Recipient	Alleles	UV^R Rou	UV^R Muc	UV^S Rou	UV^S Muc
ATC125	ATC130	F'*lon*$^+$/*lon-9*	.5	.05	.15	.3
	ATC150	F'*lon*$^+$/*lon-1*	.5	0	0	.5
ATC302	ATC130	F'*lon-9*/*lon-9*	0	0	0	1.0
	ATC150	F'*lon-9*/*lon-1*	.6	0	.2	.2
	ATC134	F'*lon-9*/*lon*$^+$	.7	.1	.2	0
ATC317	ATC130	F'*lon-1*/*lon-9*	.7	.2	.1	0
	ATC150	F'*lon-1*/*lon-1*	0	0	0	1.0
	ATC153	F'*lon-1*/*lon*$^+$	.9	.11	0	0

[a]Recombination was measured as described in section V.2, Materials and Methods. Selection was done on minimal plates lacking adenine. Merodiploids were purified by streaking and testing for mucoidy and UV sensitivity. 20 colonies were screened from each spot showing growth of recombaniants.

and UV sensitivity; the proC, and nuvA markers as well as presence of the plasmid. Fifty-three possible deletion mutants were purified twice by streaking on the same selective medium and tested for the presence of relevant genetic markers. Of these, five isolates (lon-306, lon-323, lon-328, lon-348, and lon-350), obtained from 5 independent cultures, were able to transfer the mucoid phenotype along with either the Ade^+ or Pro^+ marker to strain MC118 in plate matings. The resulting merodiploids were extremely mucoid when plated on minimal medium containing adenine and incubated at either 30C or 37C for 24 h. On the other hand plating on minimal medium lacking adenine abolished or reduced mucoidy in the merodiploids (Table XX). Furthermore, some isolates were able to transfer either Ade^+ or Pro^+ but not both.

Reversion studies proved difficult since the presumptive deleted strains were very resistant to NF and UV irradiation. Mutations lon-328 and lon-350 were considered to be possible deletions of the lon gene since the degree of mucoidy of these strains, even on complex medium at 37C, was greatly increased. It is unlikely that this is due to a mutation at the capS gene since in that case the mutation would be present in the F' donor's chromosome and would not be transferred to the F^- recipient, although a secondary mutation at this locus could enhance the degree of mucoidy in these isolates.

The fact that upon treatment with NTG, strains ATC28 and ATC50 were still extremely mucoid may indicate that at least this phenotype could not be reverted and therefore is due to a deletion. In addition, the

Table XX. Characterization of Presumptive *lon* Deletions: Ability to Transfer the Mucoid Phenotype[a]

Presumptive Deletion Strain	MS2[s][b]	Production of Mucoid Colonies[c] minimal without adenine 30C	37C	minimal without proline 30C	37C
ATC6	S	-	+	+	+
ATC8	S	-	+	M	M
ATC23	S	+	+	M	M^+
ATC28	S	-	+	+	M
ATC43	S	-	+	+	+
ATC48	S	-	M	-	-
ATC50	S	-	-	M^+	M^+

[a]Plate matings were done by the spot matings method as described in section V, Materials and Methods. Recipient: MC118.

[b]Presence of the F' plasmid was tested by cross-streaking against the male-specific phage MS2.

[c]Production of mucoid Ade^+ merodiploids. Cells were grown to mid-log phase and matings made as indicated above. Incubation was for 24 h. Symbols: S = sensitive; + = growth; M = mucoid; M^+ = heavily mucoid.

dominance pattern of mucoidy observed in some merodiploids (e.g. F' lon 328/lon$^+$) resembles the episomal dominance described for the capR9 mutation (115). It is probable that the presumptive lon deletion in strain ATC28 is really equivalent to the capR9 point mutation, although this cannot be unequivocally determined since reversion cannot be readily measured in these strains.

The difficulty in isolating deletions of the lon region may reflect the observation already noted by Chung and Goldberg (28) that the lon gene product may be essential to cell survival, since very low levels of the La protease are found even in strains which are supposedly deleted for this region. Attempts to cure strains ATC28 and ATC50 of their plasmids using acridine orange proved unsuccessful and all colonies obtained from treated cultures were mucoid and still carried the plasmid.

DISCUSSION

The different phenotypic manifestations of pleiotropic *lon*$^-$ mutations have been the subject of several independent studies, but no comprehensive work has been done in which the genetic fine structure of this locus has been directly related to the multiple functions it must control. Donch and Greenberg (42) proposed the existence of two adjacent functional units at the *lon* locus based on their sensitivity to UV. Markovitz and Baker (114) had previously presented evidence that UV sensitivity and mucoidy could be simultaneously suppressed by ochre suppressors. Subsequently, Bush and Markovitz (20) ruled out the possibility that UV sensitivity and excess formation of mucopolysaccharides were controlled by two cistrons which were part of an operon, since polarity suppressors failed to abolish either phenotype, but could not explain the multifunctional nature of the gene. In addition, the isolation and characterization of the *lon* gene product as a single polypeptide (27,28,149,189,190) argued in favor of the existence of only one *lon* gene. However, second site mutations capable of suppressing either UV sensitivity or mucoidy, but not both, have been isolated (62,69,88,89,137). Gradients in the expression of the various Lon-associated phenotypes (20) as well as different patterns of dominance for different *lon* mutations (115) have also been reported. This suggests that the pleiotropic nature of the *lon* gene could be due to either mutations affecting one or more genes, or to one gene having different domains.

This systematic study of *lon*$^-$ mutations was done with the objective of reconciling the apparent discrepancies existing between the nature of the genetic structure of the *lon* locus and its pleiotropic character, in order to understand better the molecular mechanism of control by the *lon*$^+$ gene. The evidence reported in this work indicates that the *lon* locus probably consists of one gene whose product is a multifunctional polypeptide having at least two functional domains. This can be concluded from the complementation data and the phenotypic characterization of ten independently isolated *lon*$^-$ mutants. In the discussion that follows, the results are interpreted in terms of a single *lon* gene having one polyfunctional product.

The order of *lon*$^-$ mutations within the locus was determined by cotransductional mapping using bacteriophage P1 in a way similar to that used by Kelley (94) to define the genetic fine structure of the *polA* locus in *Escherichia coli* K-12 and also used in part by Yanofsky (55,160,187) in his genetic analysis of the tryptophan operon.

The appearance of intermediate UV^R Muc (Class A) and UV^S Rou (Class B) phenotypic classes among Pro$^+$ transductants of strain MC118 was initially interpreted as the result of the separation of two genes. Subsequent detailed genetic and phenotypic analysis of the intermediate classes obtained from a P1(MC102) and a P1(AB1899) donor, reinforced the view that they were not due to unstable, segregating Lon$^+$ and Lon$^-$ clones. Though UV-sensitivity segregated clearly, mucoidy for Class A and Class B Lon$^-$ strains was intermediate. However, it must be

emphasized that the genetic separation of Lon phenotypes observed in these intermediate Lon$^-$ strains could be due to closely linked suppressor loci or may represent variations in the expression of those phenotypes tested. Further work should focus on ruling out the possibility of the existence of a closely-linked suppressor before the hypothesis of the existence of two different genes at the lon locus is discarded.

Another probable explanation for the segregation of mucoid and nonmucoid clones is the possible segregation of partial heterogenotes in transductants. Kelley (94) observed the same phenomenon while studying the fine structure of the polA locus of Escherichia coli K-12. The formation of abortive transductants seems to be inherent of P1 transduction systems; however, determination of linkage data is little affected by it (94). The analysis of segregation behavior of mucoid and rough colonies in intermediate Class A and Class B Lon strains indicate that some segregation may be caused in isolated cases by the formation of partial heterogenotes, but that these eventually stabilize. Furthermore, stable P1-sensitive derivatives are always obtained which continue to segregate mucoid and rough clones. Whether this phenomenon is due to some specific mechanism, as in the case of phase variation in Salmonella (52,191), cannot be determined from these results. The alternative remains of a possible defect in the repression system of the gal operon which could modify its interaction with the lon gene product.

Even though a certain degree of accuracy can be achieved when utilizing cotransductional frequencies for fine structure mapping, the order of alleles given here should be considered as tentative. Nevertheless, when comparisons are made of cotransduction frequencies between *lon* alleles with *proC*$^+$ obtained with either strains MC118 (F$^-$) or χ537 (F') were used as recipients. The order of the mutations is essentially the same although slight discrepancies are seen. Further detailed analysis was hindered by the difficulties encountered in generating deletion mutants of this region using nitrous acid or acridine half mustard (ICR-191) mutagenesis.

In addition, whenever three point crosses were attempted, results were unsatisfactory; perhaps due to the failure in obtaining the large numbers of transductants needed to measure accurately small intracistronic recombination frequencies.

Deletions in *lon* may affect the expression of some phenotypes but not others as evidenced from the ability of plasmid, pJMC30, in which the *lon* gene is deleted, to produce shortened polypeptides that conferred a variable nonmucoid phenotype in transformation experiments. Isolation of deletions proved difficult and although a presumptive deleted *Lon*$^-$ strain was isolated and partially characterized, the difficulty in studying its reversion capabilties do not rule out the possibility of it being due to a point mutation. It may well be that, as previously suggested by Chung and Goldberg (28), the *lon* gene product is essential for the cell's survival.

Two "clusters" of *lon*$^-$ mutations were found at opposite ends of the locus. Lon$^-$ mutations which confer the highest degree of UV sensitivity and mucoidy (*lon-9*, *lon-10*, *lon-20*, *lon-300*, *lon r-1*), are weakly linked to *proC*, whereas the mutations that map closer to *proC* are those which confer reduced expression of the Lon-associated phenotypes and perhaps represent a group of "leaky" Lon mutants (*lon-1*, *lon-6*, *lon-7*, *lon-21*, *lon t-2*). This observation is presented graphically in Figure 25. These results are similar to those reported by Donch and Greenberg (42) for their Class A and Class B UV sensitive Lon$^-$ mutants. Nevertheless, the data cannot be interpreted as the result of the existence of two adjacent genes since the two "groups" of Lon mutants remain more UV^S than the Lon$^+$ parent. It is possible that the variation in phenotypic expression observed among the different Lon$^-$ mutants and between "clusters" of alleles, reflects their influence within the locus upon phenotype. Thus, mutations found towards the promoter region could have polar effects, while mutations that lie towards the center and the promoter distal end of the locus could affect the expression of individual functions. Sivov, *et al*. (155) have reported that a point mutation of the frameshift type "+" at the *lon* locus can be suppressed by secondary point mutations of the "-" frameshift type that are located at the distal end as compared to *purE*. The authors also indicated that the *lon* gene is transcribed counterclockwise with respect to the *E. coli* genetic map.

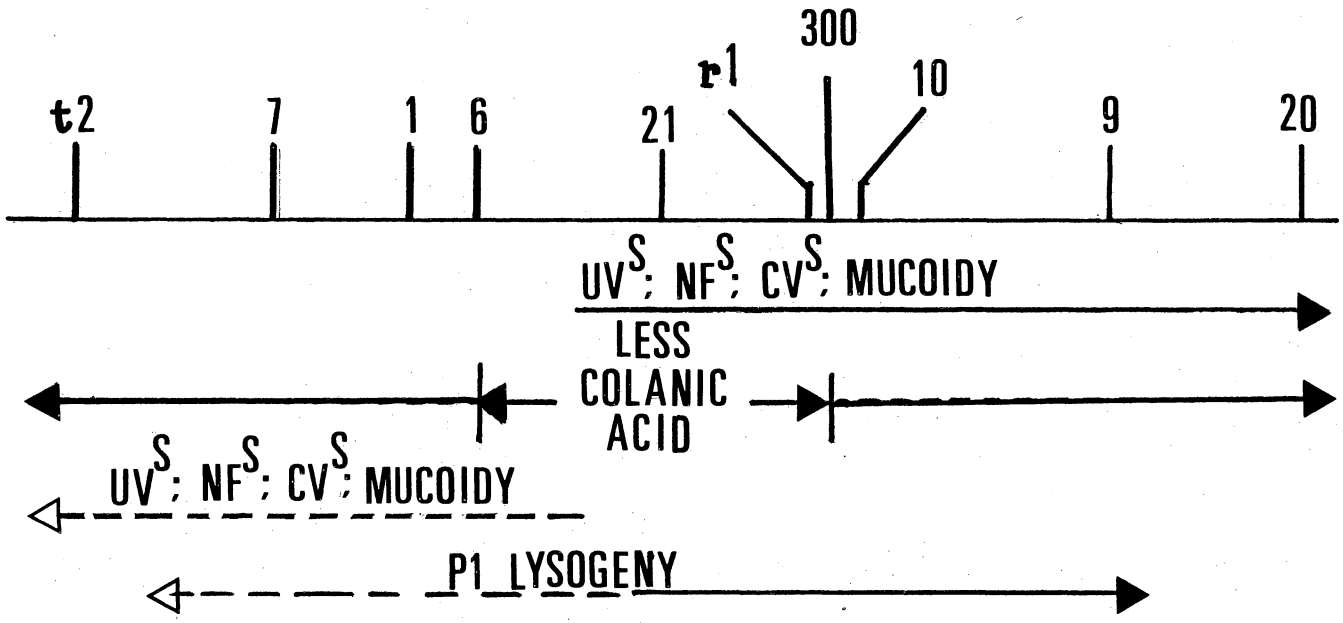

Figure 25. Correlation between map position of lon alleles and the expression of Lon-associated phenotypes. Dark arrowheads: increased expression of the lon defects. Broken arrows: decreasing degree of expression.

Bush and Markovitz (20) observed that the order of the *lon*$^-$ mutations on the genetic map could define a gradient in the expression of UV sensitivity. The results of the phenotypic characterization experiments confirm the existence of such a gradient and further extend this phenomenon to include a number of other Lon-associated phenotypes. As shown in figure 25, gradients in the expression of other Lon-associated phenotypes were also observed. A correlation exists among all phenotypes, and with the exception of mucoidy, all *lon* defects are related to UV^S to different extents. Therefore, complex medium-induced killing, filamentation, sensitivity to CV and NF as well as lowered inheritance of prophage are generally more weakly expressed by strains carrying *lon*$^-$ mutations closely linked to *proC*. Sensitivity to NA is not correlated to the intracistronic ordering of the alleles and seems to be dependent on the nature of the growth medium used. Thus, the direction of increasing degree of expression of the defective Lon$^-$ phenotypes is similar to that found by Bush and Markovitz (20) with increased expression of Lon$^-$ phenotypes being caused by the *lon*$^-$ mutations which showed the weakest linkage with *proC*. These results are consistent with the cotransduction data obtained for this region.

All Lon$^-$ strains tested in this study formed long non-septate filaments upon exposure to UV light. In addition, the introduction of all *lon*$^-$ mutations into the same genetic background abolished the spontaneous filamentation observed in some of the original Lon$^-$ strains (e.g. SG4009, JF50, MC102). That the defect in cell division caused by

the presence of the *lon*$^-$ mutation in these strains is independent of the presence of UV-induced DNA damage, is reflected in the ability of all Lon$^-$ strains tested to form filaments and lyse when shifted from minimal to complex medium, an observation previously noted by several investigators (62,178). Perhaps, the cell division defect characteristic of Lon$^-$ mutants is more dependent on "internal" metabolic signals in the cell, that could be the result of changes in pools of essential metabolites, whenever disturbances in growth conditions occur. The absence of the protease La activity in a Lon$^-$ cell interferes with the cell's ability to cope with certain physiological changes and results in the anomalies characteristic of a Lon$^-$ phenotype, even in the absence of any DNA damaging treatment. The extreme sensitization of Lon$^-$ cell to UV irradiation, reflected in the increased formation of filaments, could then be interpreted as an enhanced response of these strains to the additional metabolic stress.

DNA replication and cell division are tightly coordinated in *Escherichia coli* K-12 (74,125), and any treatment able to uncouple these two processes in a Lon$^-$ strain will result in filamentation (63,97,178) as do other treatments. The results of the experiments done in which Lon$^-$ strains were exposed to such treatments as incubation with nalidixic acid (NA) (91) crystal violet (CV) (177) and nitrofurantoin (NF) (120) indicate that, even though different patterns of sensitivity to these agents are evident among the different Lon mutants (e.g. especially NA^R among Class A and Class B Lon$^-$ transductants) filament formation occurs in all cases.

Kantor and Deering (91) noted that NA causes a metabolic imbalance which results in a reduction of DNA synthesis compared to that of RNA and protein, and leads to formation of filaments in Lon^- strains. The target of nalidixic acid in the cell is the topoisomerase DNA gyrase, an enzyme known to couple ATP hydrolysis to the introduction of negative superhelices into DNA, important in the separation and unwinding of the double helix necessary for DNA replication and recombination to occur (51,57,120,163,173). Inhibition of the ATP hydrolysis function of DNA gyrase by NA interferes with DNA synthesis; thus, causing a period of unbalanced growth (91). The results obtained when Lon^- strains were treated with NA indicate that some Lon^- mutants are more sensitive than their Lon^+ parent whereas others seem to be more resistant in minimal or complex medium (Table VIII, Fig. 8). The response depends on the growth conditions used and in most cases filamentation can be abolished and survival increased when cells are treated with NA in minimal liquid medium. It has been reported that $\underline{lon}^-$ cells exhibit slower growth rates when grown in complex media compared to $\underline{lon}^+$ cells, but that this difference is abolished when $\underline{lon}^-$ and $\underline{lon}^+$ cells are grown in ML medium (62). The results obtained indicate that the Lon^- mutants studied can be classified into three groups according to their degree of sensitivity to NA in minimal and complex medium and it can be concluded that these three groups of mutants represent three different regions of the $\underline{lon}^+$ gene in which a lesion may result in a slightly different modification of the same phenotype. This was further suggested by the observation

that Lon Class A (UV^R, Muc) mutants derived from strains MC102 (*lon-9*) and AB1899 (*lon-1*) are resistant to NA in minimal medium but sensitive in complex medium, whereas the opposite is true for Class B (UV^S Rou) mutants. It is also suggested that enhanced mucoidy of Lon^- strains in minimal medium may provide a protective mechanism that allows for increased survival of these strains. This situation could be similar to the one observed in excessively mucoid Lon^- strains in which increased production capsular polysaccharide provides protection against the lethal effects of UV irradiation (43).

It has also been suggested that UV sensitive mutants of *Escherichia coli* K-12 are more resistant to nalidixic acid than the UV^R parent (91). Among the 10 Lon^- strains tested, those that are slightly more UV^R are also more NA^R than the Lon^+ parent in minimal medium. Walker and Pardee (176) reported that at least in the Lon^- strain (PAM401; *lon-20*), nutritional repair after UV irradiation did not occur. This could indicate that the *lon* mutation in this strain not only renders it unable to restore normal cell division after exposure to UV but also abolishes its ability to overcome this inhibition under conditions known to restore the normal phenotype in other Lon^- strains. The most UV^S Lon^- strains studied failed to divide normally when treated with NA in minimal medium, indicating that the capacity to adapt to growth conditions that promote division may be dependent on the degree of UV sensitivity in these strains which is perhaps enhanced by an inability to recognize a specific signal in the cell. It has been suggested that

restoration of the DNA/Mass ratio after a period of unbalanced growth is a requirement for cell division to occur and that in Lon$^-$ cells the prolongation of this period usually results in cell death (104,178). Probably in those Lon$^-$ strains that show a decreased response to nutritional repair, failure to respond to additional changes in the cell after the restoration of the DNA/Mass ratio reduces the efficiency of the mechanism involved in promoting cell division.

That the most NA^R Lon$^-$ strains were also more sensitive to complex medium-induced killing and UV-irradiation argues in favor of the existence of a common mechanism of control. The extent to which cell division resumes depends not only on the agent causing the initial DNA/Mass ratio inbalance, but on the ability of the Lon$^-$ strains to recognize the restoration of the normal DNA/Mass ratio. The data suggests that some *lon*$^-$ mutations cause an additional impairment in NA treated strains (metabolite pools) that prevents them from dividing under growth conditions known to restore cell division on Lon$^-$ cells.

That the interaction of pools of metabolites, especially nucleotides and nucleic acids, may regulate the activity of the *lon* protease *in vivo* has been suggested by Charette *et al.* (27). Studies of the effects of nucleotide levels on cell division and prophage induction in *Escherichia coli* strain T44 a (λ), temperature-sensitive mutant done by Ruff *et al.* (143), indicated that no significant changes in the levels of ATP or cAMP occur in relation to filament formation or prophage induction. Subsequently however, Kirby *et al.* (97), showed

that in the same mutant, addition of adenine promotes prophage induction and filament formation with guanosine and cytidine reversing those effects, but that variations in the pool sizes of these molecules in strain AB1899 (lon-1) had no effect on cell division and prophage induction. In contrast to this latter result, the data gathered in this work indicate that nucleotide pools may play an important part in the control of cell division in strains AB1899 (lon-1) and MC102 (lon-9). Addition of guanosine and cytidine promotes division of the isogenic lon$^-$ strains even in the presence of nitrofurantoin, which causes breaks in DNA and inhibits the translation of inducible genes by blocking initiation of mRNA transcription (38,57). Further evidence of the direct involvement of nucleotides in the cell division process of Lon$^-$ strains (and indirectly, in the response to recovery from filamentation) comes from the behavior of Lon Class A (UV^R, Muc) and Lon Class B (UV^S, Rou) upon simultaneous exposure to NF and guanosine and cytidine. NF abolishes cell division in both Class A and Class B Lon strains, but upon addition of guanosine and cytidine division is restored with more efficiency in Class B (UV^S, Rou) strains, regardless of the lon$^-$ donor allele. By contrast, when the direct effects of guanosine and cytidine are considered, the improvement in growth observed depends on the lon$^-$ allele since growth of strains ATC151 (lon-1A) and ATC132 (lon-9B) is enhanced, while the opposite holds for strains ATC150 (lon-1B) and ATC131 (lon-1A).

Furthermore, when the survival of strains ATC151 (lon1A) and ATC152 (lon1B) to NF exposure was determined, the Class A strain was not able to recover from the division block whereas Class B exhibited slow recovery after a 90 minute lag period. This response is similar to that observed when these strains were treated with NA. These results are best interpreted as indicating that different lon mutations result in the differential expression of the same Lon-associated phenotypes and that while all lon$^-$ mutations sensitize cells to agents which interfere with DNA synthesis, some Lon$^-$ mutants are able to recover from the division block better than others implying a defect in the ability to recognize changes in a recovery signal. Clearly, this observation could only be due to a comparative study in a isogenic set of strains, where effects of different genetic backgrounds are minimized. Fluctuations in the available pools of nucleotides could very well constitute a "signal" regulating such changes. Because of the dependence of protease La on hydrolysis of ATP for its proteolytic function to be expressed (180) reduced availability of adenine in relation to other nucleotides (e.g. guanine), could be a limiting factor.

The sizes of nucleotide pools have also been implicated in the control of capsular polysaccharide production in Lon$^-$ strains (105,106). All isogenic Lon$^-$ strains studied, including Class A Lon MC118 transductants, produce mucoid colonies on minimal medium at 37C and 30C, although to different degrees. The amount of mucopolysaccharide produced, depends on the temperature of incubation and the presence of

either thymine or adenine in the medium because Lon$^-$ strains show a reduction in the degree of mucoidy when plated on minimal medium containing either nuceotide and incubated at 37C (data not shown). This finding gives further support to the observation noted by Lieberman and Markovitz (105,106) that certain nucleotides could be essential for the expression of the mucoid phenotype in Lon$^-$ strains. Because the synthesis of capsular polysaccharide, and in particular of colanic acid which is overproduced in Lon$^-$ mutants, depends on the sugar-nucleotide precursors UDP-glucose, UDP-glucuronic acid, UDP-galactose and GDP-fucose, it would be attractive to postulate a direct effect of the levels of purines and pyimidines in the cell that could interfere with capsule formation. Reduction in capsular polysaccharide production could be due to inhibition of the synthesis of either the sugar nucleotides or the nucleotides themselves. The fluctuations in the degree of mucoidy observed in most Lon strains and caused by the availability of nucleotides, is not related to the segregation of mucoid and nonmucoid colonies usually observed in Lon strains. The phenomenon occurs regardless of the presence of thymine or adenine. Furthermore, Class A (UV^R Muc) and Class B (UV^S, Rou) express their phenotypes under all conditions tested. No study on inhibition of protease La by other nucleotides has been reported.

It has recently been reported that NF elicits the stringent response in Escherichia coli K-12 (108), which results in the accumulation of the guanine nucleotides ppGpp and pppGpp (9,23,38,141)

with a concomitant decrease in the levels of nucleotides (e.g. ATP, CTP,GTP) in the cell. The possible role of the stringent response in the control of division of Lon^- strains was not investigated, but the effect of NF on growth of Lon^- mutants may be due to an arrest in protein synthesis, since it interferes with the action of RNA polymerase (135,145), leading to an increase in the DNA/Mass ratio.

The strongest evidence arguing in favor of the lon gene having a multifunctional gene product comes from the data obtained in complementation analyses in crosses between all 10 lon mutants studied. Contrary to what would be expected if two genes were present at the lon locus, the complementation behavior of lon mutations indicates that overlaps exist between segments in which complementary and non-complementary mutants are found. This is reflected by the fact that the two most accurately mapped mutations, lon-1 and lon-9, which lie in opposite mutation "clusters", complement each other. Furthermore, although the recombination frequency between the lon-1 and lon-9 alleles could not be accurately determined, preliminary data indicates that UV^R Rou (Lon^+) recombinants are found among the progeny of crosses between strains carrying these two alleles. These results are also compatible with the idea of one lon gene having two independent domains. Mutations along the length of the gene would cause differential expression of the functions controlled by lon^+ with the effect of each mutation being dependent on its position in the gene.

Examples of single genes having multiple domains such as the dur locus in the yeast S. cerevisiae which codes for both urea carboxylase activity and an allophanate hydrolase catalyzing activity that carry out in vivo degradation of urea in this organism (32); the lac repressor gene (93,134,146,192,208) and the lacY gene which defines the functional organization of lactose permease in E. coli (122) are well documented. Furthermore, overlapping genes, which produce more than one gene product from the same coding sequence have been described in bacterial and animal viruses and at least for the cheA locus in Escherichia coli (157). Examples of homomultimeric enzymes showing positive and negative complementation have been reported in E. coli (e.g. alkaline phosphatase) (55). It is probable then, that the lon gene mutations define a functional organization of the protease La in a fashion analogous to the one present at the dur locus in S. cerevisiae and the lacY gene of Escherichia coli. Furthermore, even though the existence of overlapping genes at the lon locus seems unlikely, this possibility should not be ruled out since only one product of the lon gene has been isolated, though a cloned lon gene produces two polypeptides, only one of which is the La protease (27,28,149).

Protease La, the product of the lon gene, is a tetramer composed of identical units which is involved in the degradation of normal and abnormal proteins in the cell (Deg phenotype) (27,28). This serine protease (180) has DNA and RNA binding ability, an ATP-dependent protease activity, and is able to hydrolyze ATP, which is required for

proteolysis by this enzyme (27,28,180). The fact that these three activities reside in one molecule clearly reflects its multifunctional nature. Furthermore, the actual separation of functions in a capR9 mutant that still maintained the Deg$^-$ phenotype and its DNA binding activity, although the ATPase-dependent protease and ATP hydrolysis activities had been abolished (27), argues in favor of the existence of at least two different domains controlled by the lon locus.

The ability of the lon gene product to control protein degradation in the cell (27,28,65,154,180,193), implies an important role for this protease in such cellular processes as control of gene expression, cell growth and differentiation, maintenance of a steady state in the cell and overall regulation of the cell's metabolism. Lon mutants exhibit gross metabolic defects such as the uncoupling of cell division from DNA synthesis (63,97,104,174,178), especially when exposed to treatments which damage the cell's DNA, that can be explained by the inactivation of one or more functions of this important regulatory protease. Thus, the effect of UV irradiation on lon$^+$ and lon$^-$ mutants is the same; however, it is either the presence or absence of the Lon protease activity in these strains the determining factor in the cell's division response.

From the foregoing discussion it is clear that the lon gene product exhibits functions that are not immediately related and whose expression is differentially affected under several conditions. If this is indeed the case; then, the relative frequency and "clustering" of nonsense

mutations that abolish the expression of either domain could help in the ordering of the regions defined by them within the gene. In addition, the fact that some phenotypes associated with lon can be separated by the action of different nonsense suppressors is compatible with the idea of two domains of one gene. Thus, it has been reported that most lon mutations are of the nonsense type (20,155). The results of reversion analyses presented here, although not conclusive, indicate that all lon$^-$ mutations are point mutations of the nonsense or missense type. The appearance of NF^R revertants that were also UV^R but retained the mucoid phenotype indicates that these were second site revertants (possibly similar to sul mutations). Alternatively, all could have been either suppressed strains or strains that carried small deletions on lon$^-$.

SUMMARY

The cell division inhibition defect characteristic of Lon$^-$ mutants is exhibited by all Lon$^-$ strains tested in this work. However, variations in the expression of this response are seen. The kinetics of inactivation of all Lon$^-$ strains by UV irradiation are similar and a break in the UV survival curve is observed in each case. This break is characteristic of each particular strain in the MC118 background and its magnitude dependent on their degree of UV sensitivity. The period between the exposure to UV and the break in the curve may reflect the extended division lag seen in these strains after the DNA/mass ratio is restored. The kinetics of inactivation are compatible with the existence of only one lesion at the lon locus in each strain (24,44,118).

A similar gradient in the expression of all other Lon-associated phenotypes is observed which correlates with the ordering of the lon$^-$ mutations at this locus. Treatments which cause the uncoupling of DNA synthesis from cell division are potent inducers of filamentation in all strains tested. Exposure to NA, CV and NF as well as to a nutritional shift-up results in the arrest of cell division, with the extent and sizes of filaments formed directly related to the characteristic degree of UV sensitivity of each strain.

This uncoupling of cell division from DNA synthesis is similar to that observed in recA mutants (85,86), but mechanistic differences seem to exist (36,84). All lon strains express conditional lethality during

a nutritional shift-up. It is possible that shortly after the shift-up, the rate of mass increase is accelerated while the post-shift rate of DNA synthesis is not achieved until much later (a lag of about 180 min has been reported for Lon$^-$ strains; 178), with the resulting reduction in the DNA/mRNA and protein) ratio (74). Reinitiation of cell division in Lon$^-$ cells would not occur even after restoration of the DNA/mass ratio because septum formation would be blocked by the action of an inhibitor not degraded in these strains (63,70).

Available pools of nucleotides may exert control over the action of the lon gene product as suggested previously (27,105,106). The characteristic degree of mucoidy produced by each Lon$^-$ strain can be modified by the presence of thymine or adenine in the medium. In addition, the segregation of mucoid and rough clones from mucoid colonies is observed that is not due to segregation of partial heterogenotes. Furthermore, the addition of the nucleosides guanosine and cytidine to cultures treated with NF induces the restoration of normal cell division in a fashion similar to that observed during extended treatment with the drug in minimal medium. Recently, some evidence has been presented for the involvement of the cyclic nucleotide adenosine 3',5'-phosphate (cAMP) in the induction of filamentation in Escherichia coli K-12 (170,171). In addition, cAMP and cAMP receptor protein (CRP) are known to regulate the synthesis of certain envelope proteins in this bacterium (171). The filamentation response occurs by the action of cAMP and CRP at a locus distinct from lon. Whether cAMP

exerts the same transcriptional control on the lon locus remains an open question.

The data obtained in the transductional mapping experiments indicate a "clustering" of alleles at both ends of the lon locus. Intermediate classes were recovered among Pro^+ Lon^- transductants, that exhibited "split" phenotypes with respect to UV sensitivity and mucoidy. The possibility of two genes being present at this locus was investigated since differential expression of other Lon-associated phenotypes (e.g. reduced formation of P1 lysogens, increased plating efficiency of temperature sensitive λ, sensivity to NA, NF and CV) occur in these strains. Complementation analysis of lon^- alleles ruled out the possibility of the lon locus consisting of at least two genes that form part of an operon, since overlaps are found joining complementary and non-complementary regions within the locus. This evidence gives further support to the existence of only one lon gene. Bush and Markovitz (20) presented proof that polarity suppressors had no effect on the expression of either mucoidy or UV sensitivity a fact that argues against the operon model for this locus.

The complementation and mapping data presented here is not compatible with either the existence of divergent operons, as happens with the maltose B region (76) or of overlapping genes such as is found at the cheA locus (157). The results obtained in this work argue in favor of the existence of one gene having at least two different domains and whose product is a multifunctional protein in a fashion similar to

that observed of the dur locus in S. cereviseae (32) and the regulator gene of the arabinose operon in E. coli (50). It is now known that at least three different activities reside in the lon gene product which can be separated in lon$^-$ mutants (27,28,180). Protease La is a tetramer of identical subunits (27,28) which is known to be characteristic of multifunctional proteins (55,98). In addition, usually those mutations affecting the expression of a particular activity tend to cluster in one or the other half of the map of the structural gene (98). The mapping data obtained indicate that this is the case for the lon locus. It is probable that changes in the folding patterns of different regions of the polypeptide caused by the different lon$^-$ mutations, yield distinct functional domains in this protein maybe as a result of failure to interact with other effectors (e.g. gal operon repressor).

The failure to generate deletions of the lon region and the inability to cure F' strains carrying a lon$^-$ mutation on the plasmid suggest that the lon gene product may be indispensable for the cell's survival.

Data from reversion analyses of lon$^-$ mutants, although not conclusive, partially confirms data reported elsewhere (20,155). Partial NF^R Muc revertants as well as Lon$^+$ revertants obtained were not characterized further.

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GENETIC ANALYSIS AND PHENOTYPIC CHARACTERIZATION OF LON$^-$ MUTANTS OF *ESCHERICHIA COLI* K-12

by

Angel S. Torres-Cabassa

(ABSTRACT)

A systematic study of a collection of Lon$^-$ mutants has been made in order to determine whether their pleiotropic phenotype is due to mutations affecting one or more genes. A fine structure map of the *lon* locus was constructed by P1 mediated generalized transduction. The *lon*$^-$ mutations were found to map in two "clusters" within the region. Phenotypic characterization of a set of isogenic Lon$^-$ strains derived from these experiments indicated that all Lon-associated phenotypes (e.g. sensitivity to UV irradiation, decreased ability to inherit plasmid and prophage, abnormal polypeptide degradation and regulation of capsular polysaccharide biosynthesis) are differentially expressed in Lon$^-$ strains. A direct correlation exists between the intracistronic ordering of the *lon*$^-$ alleles and the degree of expression the Lon$^-$ phenotypes in each strain.

All isogenic Lon$^-$ strains exhibit conditional lethality upon a nutritional shift-up. However, some filamenting Lon$^-$ mutants are not able to overcome this defect when exposed to growth conditions known to promote cell division in Lon$^-$ strains. Evidence was obtained that suggest a role for nucleotide pools in the control of cell division and capsular polysaccharide production.

Reversion studies indicated that all *lon*$^-$ mutations studied are point mutations. The failure to generate deletions of the *lon* region in χ573, an F' strain carrying the *lac* to *minE* region on the plasmid, and the inability to cure F' strains carrying a *lon*$^-$ mutation on the plasmid suggest that the *lon* gene product may be indispensable for the cell's survival.

From transductional crosses, two intermediate phenotypic classes: UV-resistant, mucoid (UV^RMuc), (Class A) and UV-sensitive, nonmucoid (UV^SRou) (Class B), were obtained that did not segregate colonies of the opposite morphology. Genetic analysis of these strains by back-transduction into a *proC*$^-$ *lon*$^+$ background, indicated that complete genetic separation of all Lon-associated phenotypes tested was not achieved, although differences in the expression of some of these persisted.

Data obtained from complementation analysis ruled out the presence of two genes at the *lon* locus. The patterns of complementation observed were compatible with the existence of one *lon* gene, having at least two distinct domains, and whose product is a multifunctional polypeptide.