JOURNAL OF BACTERIOLOGY, May 1993, p. 2625–2631
0021-9193/93/092625-07$02.00/0

Vol. 175, No. 9

# A Role for the Clp Protease in Activating Mu-Mediated DNA Rearrangements

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Received 28 September 1992/Accepted 21 February 1993

**Bacteriophage Mu, one of the best-characterized mobile genetic elements, can be used effectively to answer fundamental questions about the regulation of biochemical machinery for DNA rearrangement. Previous studies of Mu virulence have implicated the Clp protease in repressor inactivation (V. Geuskens, A. Mhammedi-Alaoui, L. Desmet, and A. Toussaint, EMBO J. 13:5121–5127, 1992). These studies were extended by analyzing the phenotypic consequences of *clp* alleles in two *Escherichia coli* systems: (i) the periodic replication of Mud*lac* transposons in colonies and (ii) the action of a Mu prophage in forming *araB-lacZ* coding sequence fusions. The *clpP*::*CM* mutation, which removes the proteolytic subunit of Clp protease, caused a drastic reduction in Mu activity in both systems. The *clpA*::Tn*10* mutation, which removes a regulatory subunit of Clp protease, altered the timing of Mu activity in both systems. A *clpA* deletion reduced the extent of Mud*lac* replication in colonies. These results point to temporal changes in Clp proteolysis of the Mu*cts62* repressor as a key molecular event in the regulation of one class of genomic change in *E. coli*.**

The discovery of mobile genetic elements has revolutionized our thinking about the mechanisms of genomic change (1, 14). We now consider many changes in DNA sequence organization to be the products of sophisticated biochemical systems rather than the results of replication errors or physicochemical damage (17, 20, 21). The question of how these biochemical systems are regulated has been raised by observations on the responses of various specific mutational events to physiological stress (2, 8, 11, 15). Similar questions about the regulation of genome biochemistry arise in the case of developmentally specific DNA changes.

Bacteriophage Mu is one of the best-understood mobile genetic elements at the molecular level (13). Studies of Mu-dependent rearrangements in this laboratory have involved one example of stress-induced mutation and one of developmentally specific rearrangements. (i) Coding sequence fusions between the *araB* and *lacZ* cistrons are mediated by a Mu*cts62* prophage located between them (24). These fusions do not occur under normal growth conditions but are induced by starvation stress (11, 15). (ii) *Escherichia coli* K-12 strains harboring a Mud*lac* fusion transposon in their genomes display concentric rings of β-galactosidase staining in colonies (16, 22, 23). These ring patterns result from periodic derepression of Mud*lac* replication in spatially localized cell populations during colony morphogenesis and thus constitute a prokaryotic example of developmentally regulated DNA change (23).

The Mud*lac* colony patterns are useful because they provide an easily scored phenotype for genetic analysis. The results presented here illustrate how that phenotype was used to confirm that the Clp protease (9) is an important component in the signal transduction pathway regulating Mu activation. Geuskens et al. (5) have shown that repressor molecules encoded by virulent mutants of phage Mu are much more stable in *clpP* mutants than in Clp$^+$ *E. coli* strains. Thus, it appears that Clp protease plays an important role in Mu repressor inactivation, probably by direct proteolysis. To follow up on the observations of Geuskens et al. (5), *araB-lacZ* precursor and Mud*lac* strains carrying *clp* mutations were constructed. It was found that the catalytic ClpP subunit (10) was required for the vast majority of Mu activations in both systems. In addition, it was found that mutations affecting the ClpA regulatory subunit (7) altered the extent and pattern of Mu activations.

## MATERIALS AND METHODS

**Bacterial strains.** Strain MS1534 is a derivative of M7124, an F$^-$ *thi* Δ(*argF-lac*)U169 strain; it carries the MudII1681 Mud*lac* replication-competent *lacZ* fusion element (4) near the *thyA* locus (22). At this position in the chromosome, the Mud*lac* element does not direct the synthesis of any β-galactosidase (23). The basic prefusion strain MCS2 has been described (15). Strain MCS2 was derived from MC4143 [F$^-$ *araD139 araB*::+Mu*cts62* Δ(*lacIPOZYA argF*)U169 *fla relA rpsL*] by homology-dependent lysogenization with λp1(209, U118) as described by Casadaban (3). Strains SG12049 carrying the *clpA182*::Tn*10* mutation, SG22007 carrying the *clpP1*::*CM* mutation, and SG12056 carrying a *clpA* deletion linked to a mini-Tn*10* (Tc$^r$) insertion were obtained from Susan Gottesman of the National Cancer Institute. These mutations were introduced into the MS1534 and MCS2 backgrounds by P1 transduction and selection for either tetracycline or chloramphenicol resistance. Independent transductants displayed similar phenotypes (see Fig. 2). In both MS1534 and MCS2, the Mu elements have the *cts62* allele encoding a temperature-sensitive repressor molecule, and thermal instability is preserved in *clpA*::Tn*10* and *clpP*::*CM* transductants as judged by prophage induction from MCS2 derivatives and thermal killing of MS1534 derivatives. Mu*cts62* can be plated normally on a nonlysogenic *clpP*::*CM* strain.

**Microbiological methods and photography.** Microbiological methods and photography were carried out as described previously (15, 16, 18, 22, 23). β-Galactosidase indicator agar contained the chromogenic substrate 5-bromo-3-chloro-3-indolyl-β-D-galactoside (X-Gal) at 30 μg/ml.

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## RESULTS

**Effects of *clpA* and *clpP* mutations on Mud*lac* activation in colonies.** As we have previously described, MS1534 colonies display β-galactosidase activity in concentric rings because of periodic activation of Mud*lac* replication creating functional *lacZ* fusions in spatially restricted subpopulations during growth (23). In order to test whether the Clp protease played a role in periodic Mud*lac* activation during colony development, *clpA*::Tn*10* and *clpP*::*CM* mutations were transduced into replicate MS1534 subclonal cultures and the resulting strains were inoculated onto X-Gal indicator agar together with parental controls. Inoculations were done by streaking, to yield colonies that developed from individual cells or microscopic aggregates (Fig. 1), or by toothpick stabs, to yield colonies that developed from disorganized multicellular masses (Fig. 2). The results were consistent for all transductions. In colonies arising from either type of inoculation, it can be seen that the *clpP*::*CM* mutation drastically lowered the level of Mud*lac* replication-dependent β-galactosidase expression. In contrast, the *clpA*::Tn*10* mutation altered the expression pattern. In the stab colonies, the effect of the *clpA*::Tn*10* allele on the pattern inside each colony was evident (Fig. 2). It is interesting that there was also an effect of the *clpA*::Tn*10* allele on how the positions of the colonies in the streaks affected X-Gal staining: $Clp^+$ colonies displayed most activity where farthest apart (Fig. 1a), while *clpA* colonies were most intensely pigmented where most crowded (Fig. 1b).

The weak *clpA*::Tn*10* phenotypes reported here may reflect leakiness of the insertion mutation because Western blots (immunoblots) indicated residual ClpA protein in these mutants (6a). However, tests with a Δ*clpA* allele indicated that the ClpA protein is not as important for Mud*lac* derepression as ClpP. MS1534 transductants carrying a *clpA* deletion displayed less X-Gal staining than *clpA*::Tn*10* transductants, but they still showed more β-galactosidase activity than *clpP*::*CM* transductants (Fig. 3).

The negative effect of the *clpP*::*CM* mutation was virtually absolute in the streak colonies, but the centers of the stab colonies showed significant β-galactosidase activity. Thus, as growth proceeded from the disorganized mass inoculum, there arose conditions that permitted ClpP-independent Mud*lac* replication to occur in some of the cells. Except for a few $LacZ^+$ papillae and some very faint concentric rings on the 19-day-old *clpP*::*CM* colonies, the β-galactosidase activity was limited to the colony centers. This indicates that little ClpP-independent Mud*lac* activation occurred once organized colony expansion was occurring outwards from the inoculation center (cf. reference 19). It is interesting that darker sectors eventually arose on the *clpP*::*CM* colonies.

**Effects of *clpA*::Tn*10* and *clpP*::*CM* on the kinetics of *ara-lac* fusions.** The same *clpA*::Tn*10* and *clpP*::*CM* mutations were transduced into strain MCS2 to determine their effects on the formation of *araB-lacZ* coding sequence fusions. Previous tests had shown that neither mutation affected the ability to metabolize arabinose or lactose or the ability to induce active Mu*cts*62 phage from strain MCS2 by raising the temperature to 42°C. Multiple isolates from each transduction were purified and tested by plating on arabinose-plus-lactose selective medium. The results obtained by monitoring the emergence of Ara-$Lac^+$ colonies over many days (Fig. 4) paralleled the observations on Mud*lac* replication. Fusion formation was almost completely eliminated by the *clpP*::*CM* mutation, and the *clpA*::Tn*10* mutation caused a slight but quite reproducible delay in fusion colony appearance. The colonies which did appear on the *clpP*::*CM* lawns retained the $Cm^r$ marker and were arabinose dependent for growth at the expense of lactose. Since these Ara-$Lac^+$ colonies arose after prolonged incubation, viable cells capable of producing fusions must have been present on the selective plates at very late times. We also know that fusion colony numbers are independent of the number of cells plated (15). Thus, it seems that the failure of fusion colony formation at earlier times could not easily be explained by the loss of *clpP*::*CM* viability under selective conditions.

## DISCUSSION

The observations presented in this paper confirm and extend the conclusion of Geuskens et al. (5) that the Clp protease plays a key role in Mu repressor inactivation. Geuskens et al. (5) found that *clpP* mutations retard Mu*vir* repressor inactivation in cell extracts and stabilize Mu*vir* repressors to permit stable lysogenization in vivo. My results show that the *clpP*::*CM* mutation has a very strong effect in preventing Mud*lac* replication-dependent β-galactosidase expression and MuA-dependent *araB-lacZ* cistron fusions. The *clpA*::Tn*10* mutation has a weaker effect in both systems, and a *cplA* deletion allele has intermediate effects in the Mud*lac* colony test. Since no discernible effect of the *clpP*::*CM* mutation on Mu*cts62* replication at 42°C has been observed (5), it is likely that all of these phenotypic effects are mediated via the Mu*cts62* repressor and not at some other stage of the Mu rearrangement-replication cycle.

The finding that *clp* mutations affect the Mud*lac* derepression–β-galactosidase expression patterns in colonies means that a readily scored phenotype is available for genetic analysis of the Clp protease system. The utility of this phenotype was confirmed by testing a series of 13 P1 transductants of strain MS1534 selected for the $Tc^r$ marker of a mini-Tn*10* approximately 60% linked to the Δ*clpA* mutation; there was perfect agreement between the genotypes deduced from colony staining phenotypes and those deduced from Western blot analysis of the ClpA proteins (5a). Because the colony-staining phenotype can be detected visually, a very broad range of phenotypic effects can be documented. Some phenotypes, such as lysogenization by Mu*vir* phages (5), do not permit such subtle discriminations. By analogy with other proteases (6), Clp appears to represent an important cellular regulatory system that is related to the Hsp 100 family of heat shock proteins which has been conserved in evolution (7, 10, 12). The system is assumed to be composed of a single catalytic subunit that can be combined with any of a series of alternative regulatory subunits with ATPase activity (6a, 9, 10). Thus, ClpA is thought to work through its effect on ClpP proteolysis. The different roles of the two Clp components are reflected in the distinct colony phenotypes of the *clpA* and *clpP* mutants. Removal of the ClpP catalytic subunit almost completely eliminates Mud*lac* derepression, while removal of the ClpA subunit leads to a modified but quite distinct pattern of β-galactosidase rings, suggesting that regulatory subunits other than ClpA can participate in inactivation of the fusion element's Mu*cts62* repressor. It may, therefore, be possible to identify new regulatory subunits by screening mutants with altered patterns of Mud*lac* derepression.

Both *araB-lacZ* fusions and Mud*lac* replication display temporal specificity. Fusions have been observed to occur in waves after several days of incubation on selective agar in the presence of limiting glucose (15), and the concentric rings of Mud*lac* replication reflect the formation of special-

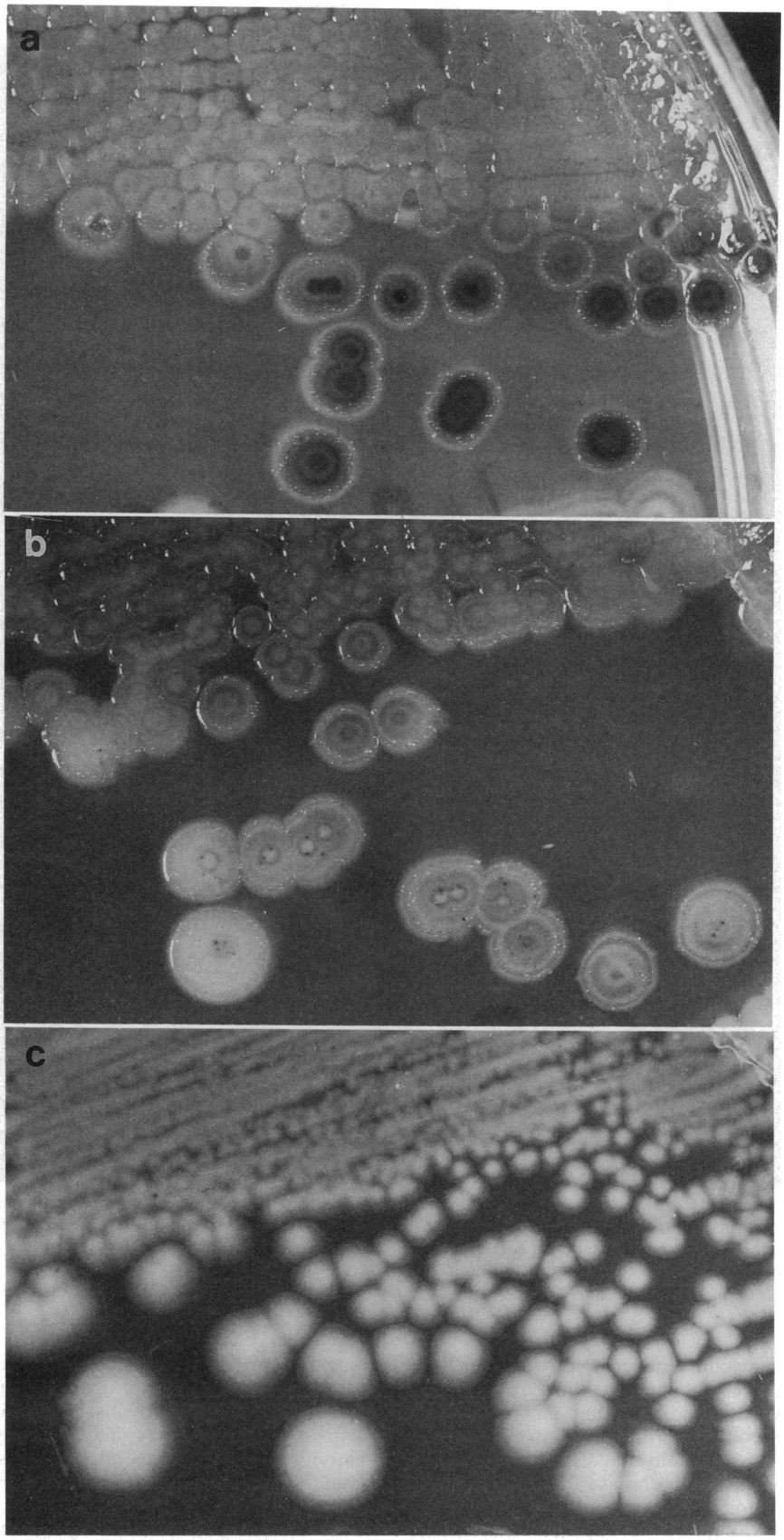


FIG. 1. Effect of *clp* mutations on Mud*lac* replication-dependent ring patterns in colonies developing from individual CFU. Cultures of MS1534 (a), a *clpA*::Tn*10* transductant (b), and a *clpP*::*CM* transductant (c) were streaked on X-Gal indicator agar and incubated for 7 days at 30°C before being photographed.

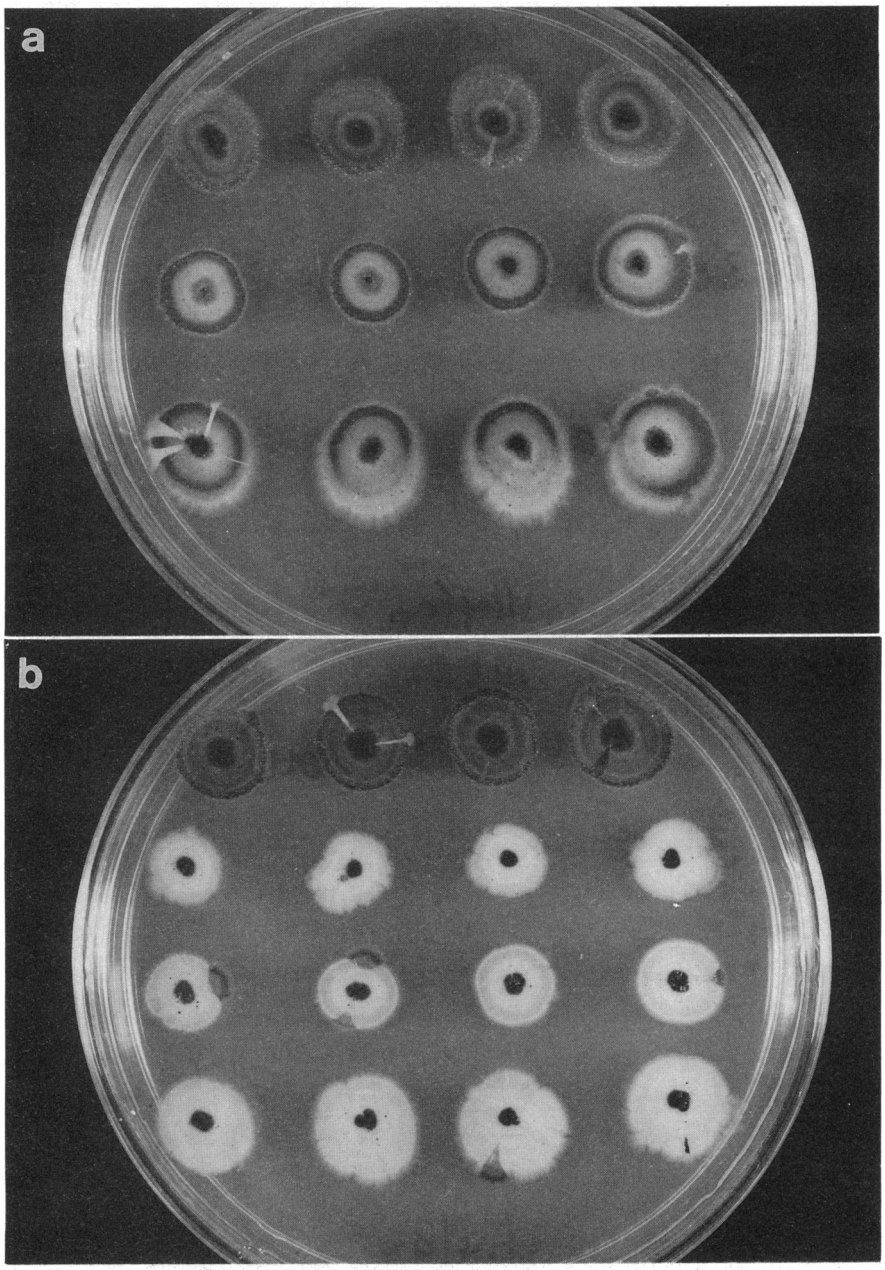


FIG. 2. Effect of *clp* mutations on Mud*lac* replication-dependent ring patterns in colonies developing from stab inoculations. Several colonies of MS1534 (top row in panels a and b), its *clpA*::Tn*10* transductants (bottom two rows in panel a), and its *clpP*::*CM* transductants (bottom three rows in panel b) were toothpicked onto X-Gal indicator agar and incubated for 19 days at 30°C before being photographed. The prolonged incubation was necessary to see the pigmentation in the sectors on the *clpP*::*CM* colonies.

ized cell populations at specific times in colony development (16, 23). The concordant results obtained with *clpA* and *clpP* mutations in both systems indicate that Clp activity is required for most of the observed derepression events. The simplest hypothesis is to postulate that Clp directly inactivates the Mu*cts62* repressor in both cases. This hypothesis suggests an explanation for a puzzle about the regulation of Mu*cts62*-based elements, namely, observations that strains with two or more copies of the Mu*cts62* control region in their genomes displayed greatly reduced Mud*lac* replication in colonies (22) and reduced, multiphasic formation of *araB-lacZ* fusions (24). These findings were difficult to understand on the basis of repressor/operator ratios, which should not change with increased dosage. However, if the Mu*cts62* repressor is periodically inactivated by a limiting proteolytic activity in agar cultures, then increased repressor amounts could saturate the protease and stabilize two or more elements. To date, all experiments on *clp* effects have been done with Mu derivatives carrying the *cts62* repressor allele. Because additional copies of either the wild-type or the thermosensitive repressor inhibit activation of Mu DNA rearrangements, extension of these observations to the

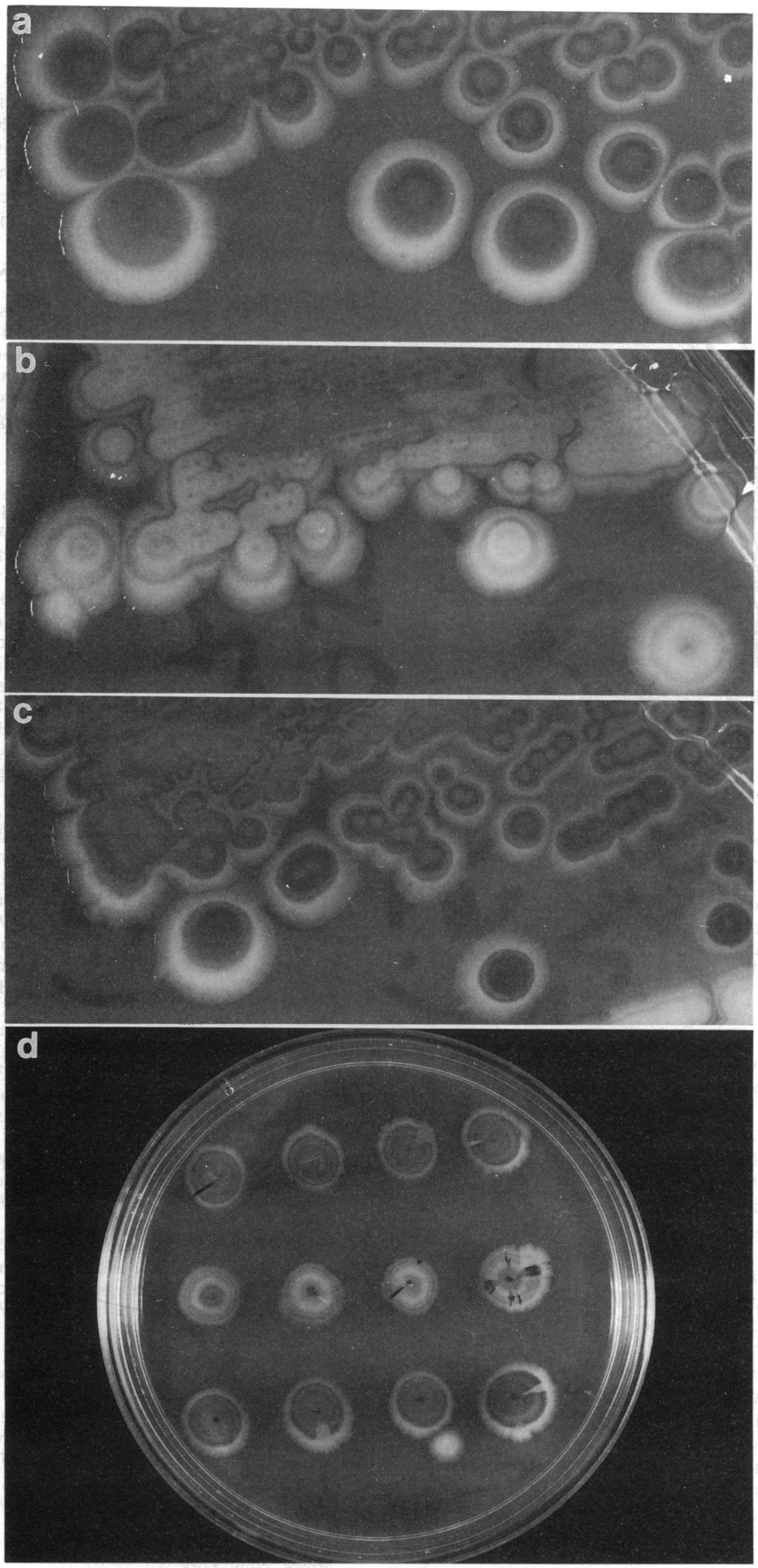


FIG. 3. Effect of Δ*clpA* alleles on Mud*lac* derepression in colonies. (a through c) Streak colonies derived from MS1534 (a), MS3287, a Tc$^r$ Δ*clpA* transductant (b), and MS3288, a Tc$^r$ *clpA*$^+$ transductant (c), after 6 days of incubation at 32°C. (d) Stab-inoculated colonies of the same three strains after 16 days of incubation at 32°C.

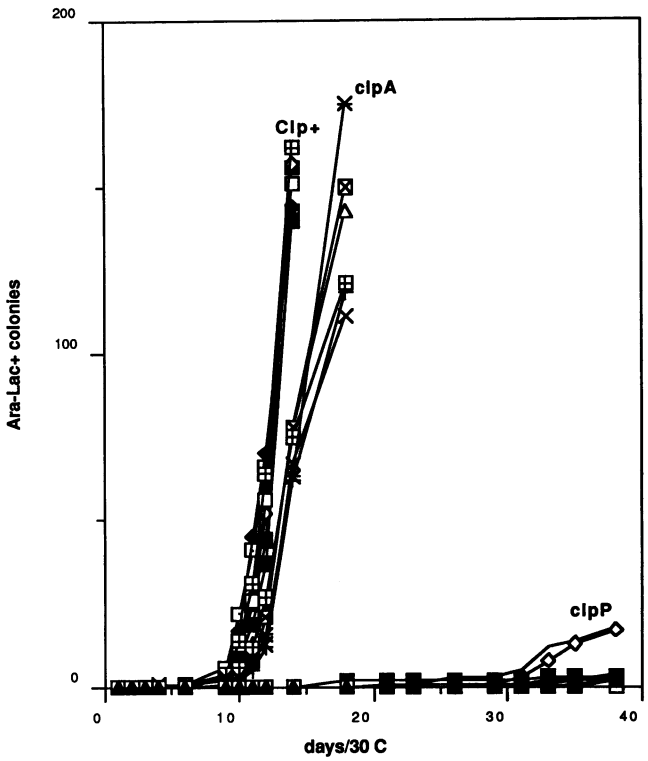


FIG. 4. Effect of *clp* alleles on the formation of *araB-lacZ* fusion clones. Cultures of the MCS2 prefusion strain (seven replicates), its *clpA* transductants (eight replicates), and its *clpP* transductants (eight replicates) were grown and plated on arabinose-plus-lactose selective agar at 30°C as previously described (15). Colonies were counted periodically, and the total number of colonies accumulated on the selective plates at each time point is indicated. Note that the data clustered according to genotype in nonoverlapping sets of curves; in particular, the kinetic difference between $Clp^+$ and *clpA* strains was quite reproducible.

$Muc^+$ repressor will have to wait for construction of Mud*lac* elements and prefusion strains carrying a single copy of the $c^+$ allele.

It is worth noting that Clp is not the only low-temperature inactivator of the Mu*cts62* repressor, because Mud*lac* derepression is clearly occurring in the centers of *clpP*::*CM* stab colonies and because *clpP*::*CM* sectors arise in which there are segments of Mud*lac* replication rings (Fig. 2). It will be interesting to learn what alternative activities are involved in these derepression events.

If, indeed, Clp is directly responsible for Mu*cts62* repressor inactivation, then either the level of the Mu*cts62* repressor or the ability of Clp to degrade it must change during bacterial development on agar. The putative changes in Clp function can be imagined to occur at many levels: transcription, translation, posttranslational modification by Clp itself or by some other activity, or incorporation into supermolecular complexes involving other regulatory factors, such as the HU or IHF proteins (13). It will be of considerable interest to explore these possibilities as examples of the developmental specificity of molecular action in a bacterial system.

The results presented here show that physiological activation of Mu-dependent DNA rearrangements requires Clp function. It is conceivable that Clp plays a role in monitoring the physiological status of the cell. If we think of transposable elements as useful agents of genomic change helping cells deal with selective challenges (20), then there need to be signal transduction systems regulating those elements in response to physiological changes. The data presented here point to Clp as a component of such a control network, linking cellular physiology and genomic stability.

## ACKNOWLEDGMENTS

I thank Michael Bailey and Teresa Reyes for technical assistance, Susan Gottesman for the *clpA* and *clpP* strains, William Clark and Michael Maurizi for doing the ClpA protein analysis of MS1534 transductants, and Lucia B. Rothman-Denes, Dan Gottschling, and Mark Hochstrasser for reading the manuscript.

This research was supported by NSF grants DCB-8816274 and MCB-9117938.

## REFERENCES

1. **Berg, D. E., and M. M. Howe (ed.).** 1989. Mobile DNA. American Society for Microbiology, Washington, D.C.
2. **Cairns, J., J. Overbaugh, and S. Miller.** 1989. The origin of mutants. Nature (London) **335**:142–145.
3. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophages lambda and Mu. J. Mol. Biol. **104**:541–555.
4. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
5. **Geuskens, V., A. Mhammedi-Alaoui, L. Desmet, and A. Toussaint.** 1992. Virulence in bacteriophage Mu: a case of transdominant proteolysis by the *E. coli* Clp serine protease. EMBO J. **13**:5121–5127.

5a. **Clark, W., and M. Maurizi.** Personal communication.

6. **Gottesman, S.** 1987. Regulation by proteolysis, p. 1308–1312. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.

6a. **Gottesman, S.** Personal communication.

7. **Gottesman, S., C. Squires, E. Pichersky, M. Carrington, M. Hobbs, J. S. Mattick, B. Dalrymple, H. Kuramitsu, T. Shiroza, T. Foster, W. P. Clark, B. Ross, C. L. Squires, and M. M. Maurizi.** 1990. Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. Proc. Natl. Acad. Sci. USA **87**:3513–3517.
8. **Hall, B. G.** 1988. Adaptive evolution that requires multiple spontaneous mutations. I. Mutations involving an insertion sequence. Genetics **120**:887–897.
9. **Katayama, Y., S. Gottesman, J. Pumphrey, S. Rudikoff, W. P. Clark, and M. M. Maurizi.** 1988. The two-component, ATP-dependent Clp protease of *Escherichia coli*. J. Biol. Chem. **263**:15226–15236.
10. **Maurizi, M. M., W. P. Clark, Y. Katayama, S. Rudikoff, J. Pumphrey, B. Bowers, and S. Gottesman.** 1990. Sequence and structure of ClpP, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli*. J. Biol. Chem. **265**:12536–12545.
11. **Mittler, J., and R. E. Lenski.** 1990. Further experiments on excisions of Mu from Escherichia coli MCS2 cast doubt on directed mutation hypothesis. Nature (London) **344**:173–175.
12. **Parsell, D. A., Y. Sanchez, J. D. Stitzel, and S. Lindquist.** 1991. Hsp104 is a highly conserved protein with essential nucleotide-binding sites. Nature (London) **353**:270–273.
13. **Pato, M. L.** 1989. Bacteriophage Mu, p. 23–52. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
14. **Shapiro, J. A. (ed.).** 1983. Mobile genetic elements. Academic Press, New York.
15. **Shapiro, J. A.** 1984. Observations on the formation of clones containing *araB-lacZ* cistron fusions. Mol. Gen. Genet. **194**:79–90.

16. **Shapiro, J. A.** 1984. The use of Mu*dlac* transposons as tools for vital staining to visualize clonal and non-clonal patterns of organization in bacterial growth on agar surfaces. J. Gen. Microbiol. **130:**1169–1181.
17. **Shapiro, J. A.** 1985. Mechanisms of DNA reorganization in bacteria. Int. Rev. Cytol. **93:**25–56.
18. **Shapiro, J. A.** 1985. Photographing bacterial colonies. ASM News **51:**62–69.
19. **Shapiro, J. A.** 1987. Organization of developing *Escherichia coli* colonies viewed by scanning electron microscopy. J. Bacteriol. **169:**142–156.
20. **Shapiro, J. A.** 1988. What transposable elements do in bacteria. Banbury Rep. **30:**3–16.
21. **Shapiro, J. A.** 1992. Natural genetic engineering in evolution. Genetica **86:**99–111.
22. **Shapiro, J. A., and N. P. Higgins.** 1988. Variation of β-galactosidase expression from Mudlac elements during the development of E. coli colonies. Ann. Inst. Pasteur (Paris) **139:**79–103.
23. **Shapiro, J. A., and N. P. Higgins.** 1989. Differential activity of a transposable element in *Escherichia coli* colonies. J. Bacteriol. **171:**5975–5986.
24. **Shapiro, J. A., and D. Leach.** 1990. Action of a transposable element in coding sequence fusions. Genetics **126:**293–299.