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Starvation-induced Mu*cts62*-mediated coding sequence fusion: a role for ClpXP, Lon, RpoS and Crp

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Summary

The formation of *araB–lacZ* coding sequence fusions in *Escherichia coli* is a particular type of chromosomal rearrangement induced by Mu*cts62*, a thermoinducible mutant of mutator phage Mu. Fusion formation is controlled by the host physiology. It only occurs after aerobic carbon starvation and requires the phage-encoded transposase pA, suggesting that these growth conditions trigger induction of the Mu*cts62* prophage. Here, we show that thermal induction of the prophage accelerated *araB–lacZ* fusion formation, confirming that derepression is a rate-limiting step in the fusion process. Nonetheless, starvation conditions remained essential to complete fusions, suggesting additional levels of physiological regulation. Using a transcriptional fusion indicator system in which the Mu early lytic promoter is fused to the reporter *E. coli lacZ* gene, we confirmed that the Mu*cts62* prophage was derepressed in stationary phase (S derepression) at low temperature. S derepression did not apply to prophages that expressed the Mu wild-type repressor. It depended upon the host ClpXP and Lon ATP-dependent proteases and the RpoS stationary phase-specific σ factor, but not upon Crp. None of these four functions was required for thermal induction. Crp was required for fusion formation, but only when the Mu*cts62* prophage encoded the transposition/replication activating protein pB. Finally, we found that thermally induced cultures did not return to the repressed state when shifted back to low temperature and, hence, remained activated for accelerated fusion formation upon starvation. The maintenance of the derepressed state required the ClpXP and Lon host proteases and the prophage Ner-regulatory protein. These observations illustrate how the *cts62* mutation in Mu repressor provides the prophage with a new way to respond to growth phase-specific regulatory signals and endows the host cell with a new potential for adaptation through the controlled use of the phage transposition machinery.

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Introduction

Transposing phage Mu is a temperate phage and can thus reside, integrated in its host genome, as a repressed prophage (for review, see Symonds *et al*., 1987). In lysogens, Mu repressor, Repc, which is encoded by the prophage *c* gene, blocks transcription from the virus early lytic promoter, pE.

Lysogens for a wild-type Mu prophage are induced upon superinfection with Mu*vir* phages that carry particular mutations in the Mu repressor gene *c* (van Vliet *et al*., 1978; Geuskens *et al*., 1991). However, no chemical or physical treatment has been found that massively induces the wild-type Mu prophage, which nevertheless undergoes low-frequency spontaneous induction. As a consequence, all experiments requiring prophage induction have traditionally been performed with thermoinducible mutants of the phage, usually Mu*cts62* (Howe, 1973). Binding to operator DNA of the *cts62* mutant repressor, which carries the single R47→Q amino acid substitution in its N-terminal DNA-binding domain, is reduced at 30°C and very weak at 42°C (Vogel *et al*., 1991).

When Mu*cts62* lysogens are grown at 42°C, the prophage is induced. Mu replication and Mu-induced chromosomal rearrangements occur at high frequency (reviewed in Toussaint and Résibois, 1983) as a result of the derepression of the phage pE promoter, which drives transcription for the early viral functions. Early transcription is negatively autoregulated by the Ner protein, the product of the first gene on the 8.2 kbp early mRNA, which also encodes the

transposition/replication functions, MuA and MuB (Fig. 1A and B). Ner also downregulates transcription from the repressor promoter pCM, probably preventing a switch from pE to pCM transcription during lytic growth (van Leerdam *et al*., 1982; reviewed in Gossen and van de Putte, 1987).

Several host factors have been shown to influence Mu immunity and rearrangement activities. These include the ClpXP protease (Shapiro, 1993; Mhammedi-Alaoui *et al*., 1994), IHF (Krause and Higgins, 1986; van Rijn *et al*., 1988; 1989; Shapiro and Leach, 1990; Alazard *et al*., 1992; Gama *et al*., 1992), FIS (Bétermier *et al*., 1993; 1995; van Drunen *et al*., 1993) and H-NS (Falconi *et al*., 1991; van Ulsen *et al*., 1996) DNA-binding proteins and the RpoS/σ^S stationary phase sigma factor (Gómez-Gómez *et al*., 1997).

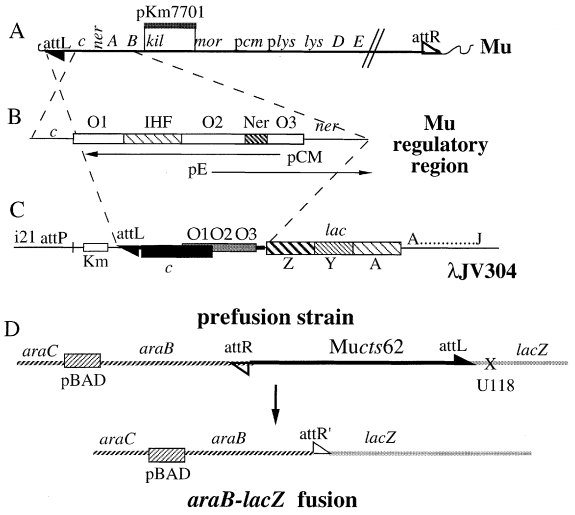

Fig. 1. A. Mu genetic map up to late gene *D. c*, repressor gene; *A* and *B*, genes that encode the MuA and MuB transposition proteins; *ner*, lytic repressor gene; *kil*, gene for the Kil protein. The Kan^r-Δ7701-1 deletion/substitution (abbreviated pKm7701) leaves the *B* gene intact but removes *kil and* several adjacent genes in the semi-essential early region of the Mu genome (4.4–10 kb from the left end).
B. Blow-up of the Mu regulatory region. O1–O3, operators; IHF, IHF binding site; Ner, Ner binding site; pE, early lytic promoter; pCM, repressor promoter; the arrows show the direction of transcription from pE and pCM.
C. Structure of the λJV304 indicator prophage. The λRS45 moiety is shown with the relative locations of the *i*21, *attP*, Kan^r and *A* to *J* λ markers; attL-O3, left-end fragment of Mu cloned in λRS45. Maps in A, B and C are not drawn to scale.
D. The *araB–lacZ* cistron fusion system. In the prefusion chromosome, the 5′ region of *araB* is separated from parallel *lac*Z and *lacY* cistrons by a Mu*cts62* prophage. This structure was obtained by introducing the λp1(209) phage, which carries a fragment of the Mu left end and *lacZY* (Casadaban, 1976; Leathers *et al*., 1979), by homologous recombination, in an *araB*::Mu*cts62* lysogen. The *lac* genes in the prefusion strain are not expressed because they lack a promoter and because there is the *U118 ochre* mutation at codon 17 of *lacZ*. Deletion removing all blocks to transcription and translation generates an *araB–lacZ* fusion encoding a hybrid arabinose-inducible β-galactosidase. In all *araB–lacZ* fusions examined, the hybrid coding sequence contained a rearranged fragment of the Mu right end (Maenhaut-Michel *et al*., 1997).

Mu transposition and Mu-induced chromosomal rearrangements also occur in cultures and colonies of Mu*cts62* lysogens kept at non-inducing temperatures (30°C or 32°C; Shapiro, 1984a,b; Shapiro and Higgins, 1989). Based on Casadaban's (1976) scheme using Mu*cts62* prophage insertions to permit the selection of *E. coli* strains synthesizing hybrid β-galactosidase proteins (Fig. 1D), the *araB–lacZ* system is remarkable because of the strict dependence of DNA rearrangements on culture conditions. *AraB–lacZ* fusions are undetectable during normal growth in liquid medium [$< 10^{-10}$ per colony-forming unit (cfu)] but form at surprisingly high levels either during prolonged incubation on selective minimal medium (MM) containing arabinose and lactose (AraLac) or after prolonged aerobic starvation in glucose MM (up to 10^{-5} per cfu) (Shapiro, 1984a; Mittler and Lenski, 1990; Maenhaut-Michel and Shapiro, 1994). This particular Mu-induced rearrangement requires MuA transposase and several host proteins, suggesting that the strand transfer complex (STC) is an intermediate in the fusion process (Shapiro and Leach, 1990). The requirement for transposase, the expression of which is repressed in Mu lysogens, suggested that aerobic carbon starvation could trigger derepression of the Mu*cts62* prophage as a first step in a regulatory cascade leading to the delayed appearance of fusion colonies on selection plates.

The experiments reported here tested this hypothesis. Using a prefusion strain (see Fig. 1D) carrying a mutated Mu*cts62* prophage that generated fusions but did not kill the host cell upon induction, we examined the effect of thermal induction of the prophage on the kinetics of fusion formation. We also tested the prediction that Mu*cts62* lysogens are induced upon long-term incubation at low temperature, using a direct indicator system of Mu derepression. In this system, the transcriptional status of the Mu early lytic promoter pE under the control of the Mu*c* repressor can be quantified directly by measuring β-galactosidase expression. The results demonstrated a significant starvation (S) derepression at 30°C of the indicator construct with the *cts62* mutation. The effect on S derepression of null mutations in two host global regulators (RpoS and Crp) and two ATP-dependent proteases (ClpXP and Lon) was analysed. Only Crp did not influence S derepression and, hence, was tested for its effect on *araB–lacZ* fusion formation.

Results

Derepression is necessary but not sufficient for araB–lacZ *fusion formation*

In order to test our prediction that prophage derepression is an important factor in determining the kinetics of *araB–lacZ* fusion colony appearance on AraLac MM, we analysed

the effect of thermal induction of the Mu prophage in prefusion strains (Fig. 1D) on the kinetics of fusion formation. Previous experiments have shown that thermal induction of the Mu*cts62* prophage in the prefusion strain MCS2 prevented fusion formation (Shapiro, 1984a; Maenhaut-Michel and Shapiro, 1994). Several *Bam* mutations (*am1066*, *am5176* and *am7154*) plus the Kan^r-Δ7701-1 substitution, which removes the Mu*kil* gene (abbreviated pKm7701) (Waggoner *et al*., 1984; Ross *et al*., 1986) were introduced in the prophage of the prefusion strain. This overcomes the lethality provoked by multiple rounds of replicative transposition and/or expression of the Kil function upon induction of the Mu prophage (Toussaint *et al*., 1987). Prefusion strains with the Mu*cts62Bam*pKm7701 prophage grew normally at 42°C on rich medium and were used to examine the effects of thermal derepression on *araB–lacZ* fusion kinetics. Exponential cultures of these strains grown and plated at 30°C or 32°C displayed fusion kinetics similar to cultures of the parental B$^+$Kil$^+$ prefusion strain, MCS2, and did not form fusion colonies until 5–10 days after plating on selective medium. In well-aerated/agitated overnight cultures at 30–32°C, MCS2 and the B$^-$Kil$^-$ lysogen behaved differently. The first formed fewer fusions, whereas the latter demonstrated accelerated fusion colony appearance (Fig. 2A).

To observe fusion formation after synchronous derepression, 32°C cultures at $OD_{600} < 0.2$ of the Mu*cts62-Bam*pKm7701 prefusion strains were thermally induced for 60 min at 42°C before plating on AraLac-selective medium, followed by incubation at 32°C. Fusion colony appearance was greatly accelerated compared with cultures maintained at 32°C (Fig. 2B). The first few colonies formed

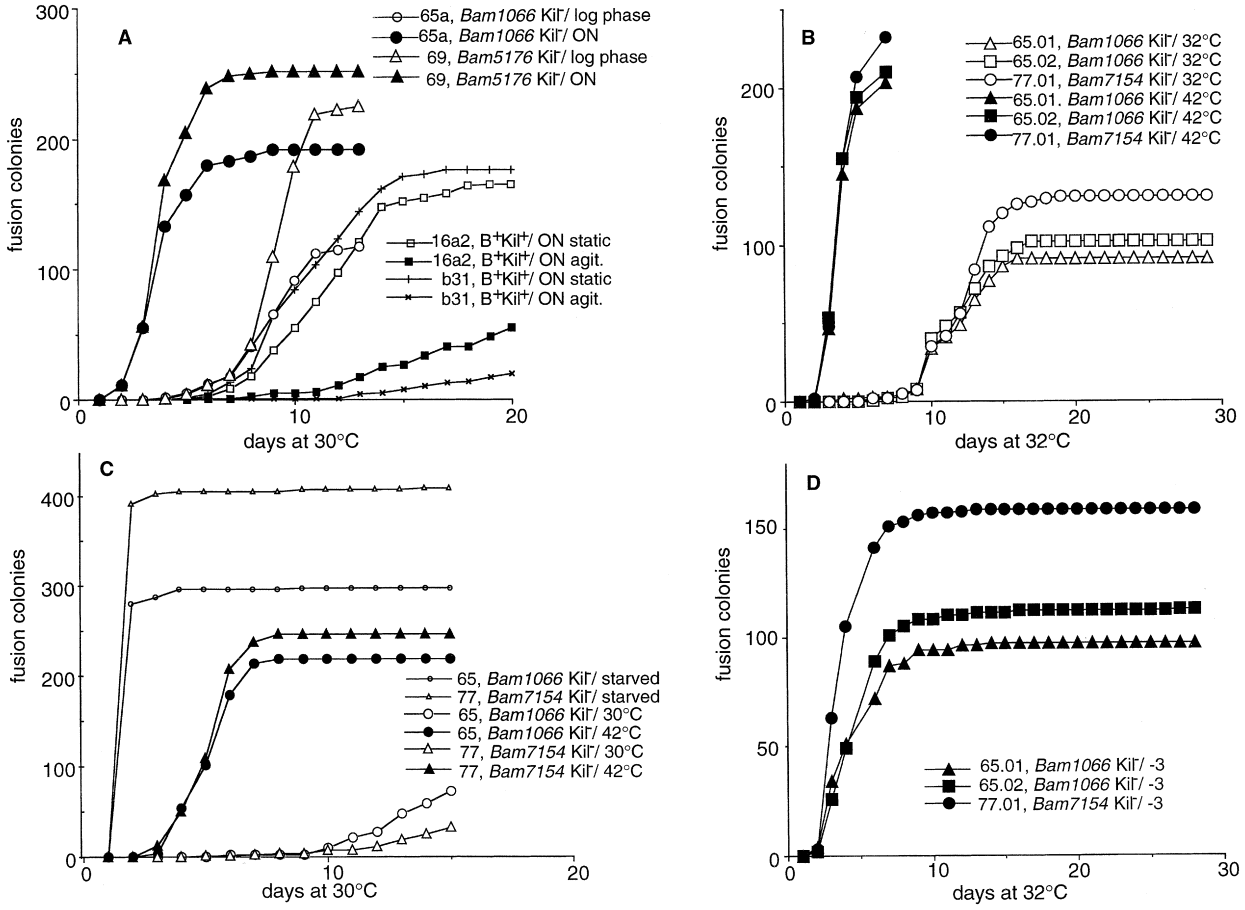

Fig. 2. Kinetics of fusion formation by repressed and derepressed Mu*cts62Bam*pKm7701 prefusion strains.
A. Well-aerated/agitated MCS1965 and MCS1969 cultures plated in log phase (open symbols) or after S derepression by overnight growth to saturation (closed symbols). Unaerated/static cultures (open square and +) and well-aerated/agitated (closed square and x) cultures of MCS2Mu*cts62*B$^+$Kil$^+$ prefusion strains (16a2 and b31) plated after overnight growth to saturation.
B. Three independent cultures (MCS1965.01, 1965.02 and 1977.01) plated immediately after 60 min incubation at 42°C (closed symbols) as well as after incubation at 32°C (open symbols).
C. MCS1965 and MCS1977 cultures after 10 days of aerobic starvation in minimal glucose (0.4%) medium (small open symbols), after 60 min induction at 42°C (large closed symbols) or after incubation at 30°C (large open symbols).
D. The three cultures shown in (B) induced at 42°C for 60 min and plated after 10^{-3} dilution in fresh LB and overnight static incubation at room temperature.

by the induced cultures already appeared on days 2 or 3 after plating. New colonies continued to appear daily until the capacity of the plate was saturated. Moreover, the rate of fusion colony accumulation was clearly higher for the thermally induced cultures (Fig. 2B). No variation in behaviour was observed between strains carrying different *Bam* alleles (Fig. 2 and data not shown). Repressor inactivation thus played a significant role in fusion kinetics, as predicted. In the B^-Kil^- prefusion strain, the effect of thermal induction on both the kinetics (reduced delay) and the amount of fusion colonies was similar to that observed in well-aerated overnight cultures maintained at 30°C (Fig. 2A). This suggested that prophage derepression was triggered by agitation (or aeration) and growth to saturation. We called this observed induction effect 'S' derepression and will justify this term further below.

The kinetics of colony appearance on selective plates from the thermally induced cultures differed significantly from those observed with long-term starved cultures in minimal glucose medium. These have been shown previously to contain *araB–lacZ* fusions formed before plating on selective medium (Maenhaut-Michel and Shapiro, 1994). They displayed over 95% of their final colony yield in the first 2 days of incubation compared with about 2% in the S and thermally induced cultures analysed here (Fig. 2C). This discrepancy indicated that the great majority of colonies produced on AraLac-selective medium inoculated with thermally induced cultures did not grow from fusions formed before plating on selective medium. When thermally induced cultures were diluted 100- or 1000-fold in fresh medium and grown overnight at room temperature before plating, the kinetics of fusion production were similar to those obtained by plating immediately after thermal induction (Fig. 2D). Here again, there was no evidence for completion of the fusion process before plating, as only very few fusions appeared in 2 days of incubation on selective plates. These observations suggested that the derepressed state, an 'activated state' for fusion formation that did not allow completion of fusion formation before selection, was maintained when the cells were returned to a non-inducing temperature.

The lag between thermal induction and completion of the fusion process meant that starvation contributed at least one additional essential signal to the fusion process in addition to provoking prophage derepression. The existence of the lag was confirmed using a replica-plating assay that allowed for the detection of fusion production in the clonal progeny of single colonies patched on LA plates (Fig. 3A and *Experimental procedures*). Patches were grown at 32°C from single colonies obtained by plating dilutions of Mu*cts62Bam*pKm7701 prefusion cultures before and after 60 min incubation at 42°C. Replicas of patches from thermally induced cultures onto selective AraLac medium containing Xgal indicator displayed no blue colonies at day 2 of incubation, indicating the absence of pre-existing *araB–lacZ* fusions. However, they produced blue colonies earlier (days 3–6) and in greater numbers than replica patches originating from the control culture maintained at 32°C, which produced sporadic blue fusion colonies from day 6 onwards (Fig. 3B). In reconstruction experiments, pre-existing fusions were introduced in the patches, at a frequency of about 1 per 10^6 cfu of the prefusion strain. The expected number of blue fusion colonies appeared on the replicated patches after 2 days of incubation, indicating that fusions formed at frequencies as low as 10^{-6} before transfer to the selective medium could be detected. Using a similar assay, we determined the proportion of derepressed individuals among the culture population by measuring their capacity to produce fusions. Between 33% and 80% of the cells in vigorously aerated overnight cultures at 30°C were activated for fusion production and had thus undergone S derepression (Fig. 3C).

The absence of *araB–lacZ* fusions in thermally induced (60 min at 42°C) cultures of Mu*cts62Bam*pKm7701 prefusion strains was also confirmed by direct examination of their DNA by polymerase chain reaction (PCR) for DNA junctions diagnostic of *araB–lacZ* fusions (data not shown).

Western blot analysis was used to search for Rep*cts62* protein in crude extracts prepared from aliquots of thermally induced (60 min) Mu*cts62Bam*pKm7701 prefusion cultures after growth at low temperature (32°C) for about 10 doublings. The cultures were composed of > 97.5% activated cells, as tested by the replica-plating assay described above. The Rep62 repressor protein was undetectable in the thermally induced samples, but easily detectable in the uninduced controls (Fig. 4).

*Mu*cts62 *lysogens are derepressed at 30°C upon aerobic growth to saturation*

The response of Mu repression and induction to growth phase was analysed using a direct indicator system of Mu derepression that consists of a Δ*pro,lac E. coli* strain, lysogenic for a single copy (inserted at the λ *att* site) of λRS45 (Simons *et al*., 1987) derived prophages with the left end of Mu. They contain the complete Mu repressor gene *c*, the repressor promoter pCM and the early lytic promoter pE, all of them in their natural configuration, but no functional *ner* gene. pE is fused to a cryptic *lac* operon, so that *lacZ* replaces the Mu early lytic genes. It is regulated, *in cis*, by the Mu repressor expressed from its natural promoter, pCM, so that the Mu*c* repressor coding sequence is subject to negative autoregulation (Fig. 1C; Vogel *et al*., 1991). We used MC4100 (Δ*pro,lac*) lysogenic for λJV300, λJV304, λJV313 or λJVc. These four indicator prophages carry the wild type, the *cts62*, the *cts62*,*sts62-1* and a null allele of the Mu*c* gene respectively. The *sts62-1* mutation

restores the activity of the *cts62* repressor at high temperature (Vogel *et al*., 1996). Flasks containing LB were inoculated with cultures grown with or without aeration and incubated at 30°C with vigorous aeration for various intervals before sampling for β-galactosidase assay. Table 1 A shows that the λJV304 (*cts62*) lysogen went from less than 10 Miller units during log phase to about 800 units after overnight incubation. This result indicated that a fraction of the population was induced by aerobic growth to saturation, confirming the existence of S derepression. No change in β-galactosidase activity could be detected in MC4100(λJV300) (c^+) (Table 1A) or MC4100(λJV313) (*cts,sts*) (data not shown). In MC4100(λJVc) (repressor null mutant), the β-galactosidase specific activity was very high (around 11 500 Miller units) as expected, although it decreased about fourfold in the overnight culture (Table 1A). This lower specific activity in overnight cultures might reflect a lower activity of the pE promoter or a lower level of protein synthesis when cells are in stationary phase.

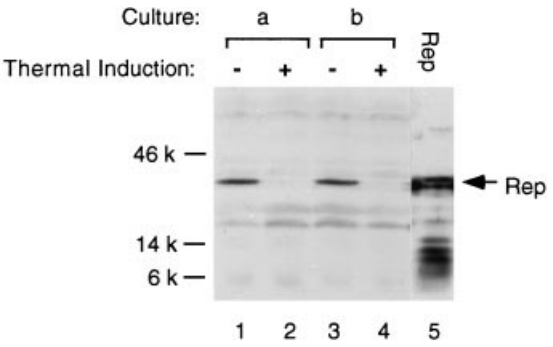

Fig. 4. Rep62 levels remained undetectable at 32°C many generations after thermal induction. Two MCS1977 cultures from two independent colonies (a and b) were grown to 1×10^8 cells ml^{-1} and subjected to thermal induction (60 min at 42°C) as indicated (lanes 2 and 4). Cultures that ranged between 1.4 and 4.6×10^8 cfu ml^{-1} immediately after thermal induction were diluted 20-fold and grown overnight to 1×10^{10} cfu ml^{-1} at 32°C. As a control, uninduced cultures were grown to the same density at 32°C (lanes 1 and 3). Repressor levels in 1×10^9 cells were examined by Western blot analysis using polyclonal rabbit antibodies raised against purified Rep62. Lane 5, 1 μg of purified Rep.

S derepression also occurred in cultures starved in MM, conditions that have been shown to activate fusion production (Mittler and Lenski, 1990; Maenhaut-Michel and Shapiro, 1994). Overnight incubation at 30°C of MC4100(λJV304) in liquid glucose MM and on minimal

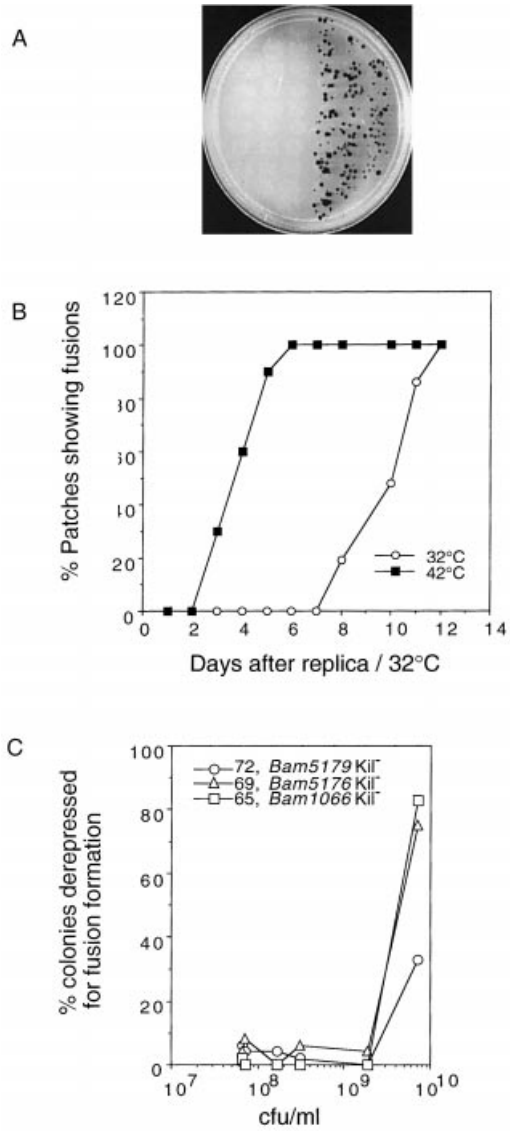

Fig. 3. Replica plate analysis of derepression for *araB–lacZ* fusion formation.
A. Replica patches from single colonies of a *Bam*pKm7701 prefusion strain before and after thermal induction. An MCS1964 culture was grown at 32°C to $OD_{600}<0.2$, diluted and plated for single colonies at 32°C, then incubated at 42°C for 60 min, diluted and plated for single colonies at 32°C. Patches were made by stabbing isolated colonies from the 32°C (left) and 42°C (right) culture on LA plate and then replica plating (see *Experimental procedures*) onto AraLac indicator medium with 0.01% glucose and Xgal. This photo was taken after 6 days incubation at 32°C.
B. The kinetics of blue fusion colony appearance on replica patches of repressed and derepressed cultures. A master plate was prepared by stabbing 40 isolated colonies after plating 32°C or 42°C cultures of MCS1977. These patches were replica plated onto AraLac indicator medium with 0.01% glucose and Xgal. The plates were incubated at 32°C, examined daily and scored for the percentage of replica patches that contained at least one blue colony. The scoring distinction between repressed and derepressed patches was complete by day 6, when 100% of the derepressed patches and 0% of the repressed patches contained fusion colonies.
C. The kinetics of S derepression at 30°C measured by replica plate assay. MCS1965, MCS1969 and MCS1972 cultures were grown with vigorous aeration/agitation in LB medium and plated on LA at intervals for single cfu. The colonies were then replica tested for fusion formation as described in *Experimental procedures*, except that the test agar did not contain glucose. The highest density samples were obtained after overnight incubation.

Table 1. Growth and host mutation effects on S and thermal induction of MC4100(λJV304*cts62*).

A. Growth culture parameters affecting S derepression. β-Galactosidase specific activities (Miller units) are the means of at least two experiments. Standard deviations were calculated where 10 independent cultures were assayed. OD_{600} was measured at the time of sampling for enzyme assay. All cultures were inoculated with a 0.01 volume of a preculture (precult), which was grown overnight at 30°C unless indicated otherwise, from one isolated colony in LB in a tube without shaking. Cultures were grown at 30°C in flasks in a shaker (aerated cultures) except when indicated ('static', i.e. without shaking). (ON) indicates an overnight culture. Ages of older cultures are indicated as the number of days of incubation.

Strain	Culture	OD_{600}	β-Gal. activity (Miller units)
λJV304 (*cts62*)	LB, aerated precult[a]	4.0 (precult)	146 ± 68
	Aerated cultures	0.5	127 ± 140
		1.2	133 ± 125
		4.7 (ON)	1038 ± 244
	LB, static precult	0.5	6 ± 9
	Aerated cultures	1.2	62 ± 12
		4.0 (ON)	800 ± 111
	LB, static precult	0.7 (precult)	28 ± 8
	Static cultures	0.5	46 ± 12
		1.2 (5 day)	41 ± 10
		1.2 (6 day)	75 ± 36
		1.3 (7 day)	178 ± 30
		1.4 (8 day)	235 ± 29
	H_2O/10% LB	0.15	53
		0.4	139
		0.7 (ON)	1734
	MM/10% LB	0.15	102
		0.4	223
		0.7 (ON)	739
λJV300 (*c*+)	LB	0.3	58
		2.4 (ON)	76
λJVc (Def)[b]	LB	0.2	11 608
		1.2	11 842
		4.0 (ON)	2911

a. Precultures aerated in a roller drum. Note that, in this case, higher background β-galactosidase specific activities are observed in early log phase cultures.
b. A null allele of the Mu*c* gene.

agar without a carbon source showed significant derepression, but weaker than in LB medium (313 Miller units in MM–0.4% glucose; 180 Miller units on minimal agar). The addition of a limiting concentration of glucose to the starvation plates stimulated derepression (180 Miller units at 0% glucose versus 500 Miller units at 0.04% glucose), just as it was observed to stimulate and accelerate the formation of *araB–lacZ* fusion clones (Shapiro, 1984a). Agitation of the cultures, which was shown to stimulate fusion production in Mu*cts62* lysogens (Maenhaut-Michel and Shapiro, 1994; Fig. 2A), also stimulated S derepression by a factor of 5–20 (Table 1A). In static cultures, in which cell density remained much lower than in well-aerated cultures ($OD_{600} = 1.2$, i.e. 1×10^9 cfu ml^{-1} compared with $OD_{600} = 4.7$, i.e. 8×10^9 cfu ml^{-1} respectively), no increase in β-galactosidase specific activity was observed before day 6.

In an attempt to distinguish between the effect of cell density and nutrient exhaustion on S derepression, pE-driven *lacZ* expression was measured in cultures of MC4100(λJV304) grown at 30°C in diluted LB (see *Experimental procedures*). In this medium, S derepression reached the same level in overnight cultures as in regular LB (Table 1A), despite the fact that colony-forming units did not exceed $1–2 \times 10^9$ cfu ml^{-1} (compared with 8×10^9 in LB). At that cellular density ($OD_{600} = 0.7$), very little derepression was observed in LB (Table 1A), suggesting that nutrient depletion, rather than cell density, triggered S derepression.

To test the possible influence of Mu functions on S derepression, we examined strains MCS2652 and MCS2653, which carried λJV304 (*cts62*) and the Mu*cts62Bam1066*pKm7701 prophage, and hence expressed Mu functions including Ner and the MuA transposase. These strains were derived from the prefusion strain MCS1965 by curing the λp1(209) prophage (UV irradiation) and introducing the indicator prophage (see *Experimental procedures*). MCS2652 and MCS2653 grew normally in the derepressed state. Compared with MC4100(λJV304), β-galactosidase activity in MCS2652 was slightly higher in exponential phase (52 Miller units) and increased upon overnight incubation at 30°C, although less than in the absence of the Mu prophage (211 Miller units). Similar results (data not shown) were obtained

B. Host factor effects on S and thermal derepression. The values shown are means of three independent measurements. Cultures were grown at 30°C, with aeration, in LB or LB diluted 1:10 in 132 minimal medium without a carbon source (MM/10% LB). β-Galactosidase activity was assayed at different growth phases, before and after 30 min incubation at 42°C, with aeration.

Strain	Medium	30°C OD_{600}	β-Gal (Miller units)	OD_{600}	42°C β-Gal (Miller units)
Wild type	LB	0.05	10	0.1	2070
		0.1	38	0.2	2530
		0.3	25	0.6	1484
		0.8	103	1.0	1764
		1.6	93	1.8	165
		1.9	127	2.0	121
		3.5 (ON)	1657	3.5 (ON)	2142
	MM/10% LB	0.2	42	0.2	3260
		0.6	113	0.6	1381
		0.7 (ON)	775	0.7 (ON)	660
lon::Tn*10*	LB	0.1	95	0.15	4479
		2.7 (ON)	61	3.3(ON)	165
	MM/10% LB	0.1	54	0.14	700
		0.4	58	0.4	1493
		0.6 (ON)	88	0.6 (ON)	246
clpP::Cm	LB	0.06	158	0.07	994
		3.4 (ON)	49	3.2 (ON)	64
	MM/10% LB	0.15	45	0.2	3964
		0.55	47	0.55	467
		0.52 (ON)	61	0.5(ON)	96
clpXam14	LB	0.2	109	0.4	4601
		3.3 (ON)	130	3.3(ON)	160
rpoS::Tn*10*	LB	0.4	65	0.7	2390
		4.0 (ON)	90	4.0 (ON)	85
	MM/10% LB	0.1	64	0.15	3706
		0.5	93	0.5	567
		0.6(ON)	150	0.6 (ON)	175
crp::Cm	LB	0.4	136	0.6	2090
		4.3(ON)	1861	4.3(ON)	1890

with strains harbouring other combinations of λ indicator and Mu prophages. Partial induction in overnight cultures at 30°C was thus not restricted to the MC4100(λJV304) indicator strain and was apparently downregulated by Ner as expected.

Thermal induction of pE repressed by the cts62 *repressor is growth phase sensitive*

In λJV304 lysogens, β-galactosidase expression is thermoinducible (Vogel *et al*., 1991). As a control for evaluating the extent of S derepression in the above experiments, we measured β-galactosidase expression in thermally induced MC4100(λJV304). Cultures were grown at 30°C with aeration and sampled at various times. β-Galactosidase was assayed after 30 min incubation at 42°C. Table 1B shows that thermal induction was very efficient, as expected, when cells were shifted to 42°C while in early logarithmic phase (below $OD_{600} = 0.3$, i.e. 10^8 cfu ml^{-1}). β-Galactosidase expression from pE was lower in cells induced while in late log phase and, in thermally induced overnight cultures, did not increase over the values resulting from S derepression. This was also the case in cultures grown in diluted LB to densities that permit thermal derepression in cultures growing in LB (Table 1B), suggesting that thermal induction was inhibited/prevented by starvation.

The experiment was repeated with the Mu*cts62-Bam1066*pKm7701 lysogen, MCS2652. Induction was observed when the cultures were induced while in log phase but, again, no further increase over S derepression could be detected upon thermal induction of overnight cultures. When cells were shifted to 42°C while in late log phase, however, β-galactosidase activity was more abundant than in MC4100(λJV304) (data not shown). Different Mu*cts62*-derived prophages (Mu*cts62*pAp1, the Ner^+, A^-, B^- mini-Mu Mu18-1 and the Ner^+, A^+, B^- Mu18A-1) were introduced in MC4100 (λJV304). The lysogens obtained were tested in the same way and provided results very similar to those obtained with MSC2652 (data not shown).

To test for an effect of cell density on the thermal induction of β-galactosidase expression, MC4100(λJV304) was grown in LB to early exponential phase and centrifuged. Cells were concentrated 25-fold by resuspension in fresh

broth and again induced at 42°C for 30 min. No β-galactosidase expression could be detected (data not shown). Thus, thermal induction seemed to respond negatively to cell density as well as to starvation.

To evaluate the effect of growth phase on pE activity, MC4100(λJVc), which expresses no active represssor, was grown continuously at 42°C. β-Galactosidase specific activity kept increasing (from 7400 to 12 000 Miller units) when the culture went from early exponential to overnight growth (Table 2). The same result was observed when MC4100(λJV304) was induced (30 min at 42°C) in early log or late log phase and grown continuously overnight at 42°C or shifted back to 30°C (from 5000 to 15 000 Miller units when induced in early log phase, and from 400 to 7500 Miller units when induced in late log phase; Table 2). pE thus retained some activity in stationary phase cultures at 42°C, which was consistent with pE activity observed during S derepression at 30°C.

S derepression requires ClpXP and Lon proteases as well as RpoS, but not Crp, which are all dispensable for thermal derepression

Because carbon starvation and various regulatory factors have been implicated in the control of *araB–lacZ* fusion formation, we introduced *clpP*::Cm, *clpXam*14, *lon*::Tn*10*, *rpoS*::Tn*10* and *crp*::Cm mutations in MC4100(λJV304) by transduction to test their effect on S and thermal derepression. None of these mutations blocked thermal derepression of log phase cultures, but the two *clp*, the *lon* and the *rpoS* mutations eliminated S derepression (Table 1B). The *crp* mutation had no inhibitory effect but rather stimulated S derepression, which thus involved RpoS, ClpXP and Lon, but not the cAMP-Crp system.

Despite the fact that the basal level of β-galactosidase remained low because of the absence of S derepression in the *clpP*, *lon* and *rpoS* strains, no increase in β-galactosidase expression could be detected in the overnight cultures induced for 30 min at 42°C (Table 1B). This reinforced the above conclusion that thermal induction of pE expression was growth phase sensitive.

Crp requirement for fusion formation in the presence of MuB protein

The results described above indicated that fusion formation required a starvation-dependent event in addition to derepression. As Crp was not required for S derepression, it was possible that the second starvation-specific control function was dependent on the cAMP-Crp catabolite control system. A *cya*::miniTn*10* mutation was indeed found to inhibit fusion formation when introduced into the standard prefusion strain MCS2, and a *crp*::Cm mutation had an even stronger negative effect (data not shown). The result was ambiguous, however, as either mutation was also expected to prevent transcription from the pBAD promoter (Saier *et al*., 1996) and hence to block expression of the AraB–LacZ fusion protein. Transcription from pBAD was

Table 2. Expression from pE in strains grown at 42°C.

Strain growth conditions	OD_{600}	β-Gal (Miller units)
MC4100(λJVc)		
Grown continuously at 42°C	0.4	7390
	1.3	9441
	(ON) 3.9	12 011
MC4100(λJV304)		
Induced at 42°C in early log phase		
After 30min at 42°C	0.4	5000
After ON growth at 42°C	3.8	14 509
After ON growth at 30°C[a]	3.6	15 179
Induced at 42°C in early stationary phase		
After 30min at 42°C	2.8	371
After ON growth at 42°C	3.1	7890
After ON growth at 30°C[a]	3.6	7315
Grown continuously at 30°C		
Early log phase	0.3	27
Entry into stationary phase	2.7	334
After ON growth (S derepression)	3.8	1495

a. Shifted back to 30°C after 30min at 42°C.

β-Galactosidase specific activities are given in Miller units. MC4100(λJVc) (repressor null mutant) was grown continuously at 42°C in LB with aeration. MC4100(λJV304) was grown in LB at 30°C with aeration, induced at 42°C either in early log phase ($OD_{600} = 0.26$) or in early stationary phase ($OD_{600} = 2.7$) and grown continuously at 42°C or shifted back to 30°C after 30 min at 42°C. As a control, an aliquot of the MC4100(λJV304) culture was kept at 30°C and grown overnight with aeration. β-Galactosidase specific activities were measured as indicated.

therefore rendered Crp independent by introducing a plasmid carrying an *araC*^{c} mutation (pXJS5677) in the prefusion strains (see *Experimental procedures*).

Table 3 shows the results of prolonged incubation at 32°C on selective AraLac medium of Crp^{+} and *crp*::Cm derivatives of *araC*^{c} prefusion strains with a B^{+}Kil^{+}, B^{+}Kil^{-} or B^{-}Kil^{-} Mu*cts62* prophage. The absence of Crp reduced fusion formation by approximately two orders of magnitude in the B^{+} strains, whether they were Kil^{+} or Kil^{-}. However, the B^{-}Kil^{-} strains showed a partial bypass of the Crp^{-} block and produced 10–60% of the total fusions produced in the Crp^{+} strains. This partial bypass was also observed after thermal induction of *crp*::Cm (Mu*cts62Bam*pKm7701) pXJS5677*araC*^{c} prefusion strains. In this experiment, the first fusion colonies appeared at day 3, and they accumulated at levels ranging from 40% to 70% of the Crp^{+} control strains (data not shown). As the presence of the pKm7701 substitution (Kil^{-} phenotype) did not influence fusion formation in the Mu*cts62*B^{+} *crp*::Cm lysogen, the bypass in the MuB^{-}Kil^{-} lysogens appeared to be caused by the absence of MuB function.

Persistence of the induced state

The results in Figs 2D and 3 provided evidence that, in Mu*cts62Bam*pKm7701 lysogens, the derepressed state after thermal or S derepression persisted through many cell divisions at 30°C. We tested whether this would apply to MC4100(λJV304). It was grown to early log phase in LB, incubated for 60 min at 42°C and then shifted back to 30°C, with and without dilution in fresh LB. β-Galactosidase was assayed at various times after the temperature downshift. Specific activity kept increasing in both cultures, although the increase was much more pronounced in the undiluted culture. Little difference was observed between cultures maintained at 42°C and returned to 30°C undiluted (Fig. 5, dotted and continuous lines respectively). However, when the cultures were diluted, β-galactosidase activity was lower in the cultures that were shifted to 30°C. This suggested that, in the undiluted cultures, most cells remained derepressed despite the shift back to 30°C, while in the diluted cultures, a significant proportion of the cells returned to the repressed state upon growth at 30°C.

The *rpoS*, *clpP*::Cm and *lon*::Tn*10* derivatives of MC4100(λJV304) were carried through the same experiments. The *rpoS* mutation did not affect β-galactosidase activities (data not shown), suggesting that σ^{s} neither affected pE activity directly nor interfered with the re-establishment of repression. The *lon* and *clpP* strains, however, displayed lower β-galactosidase activities, *lon* having a more pronounced effect than *clpP* (Fig. 5), and this effect was more prominent in the diluted cultures. Thus, in addition to being required for S derepression, Lon and Clp seemed to prevent the re-establishment of repression after return to 30°C.

To gain more information about the status of individual cells in populations that underwent thermal induction and a return to 30°C, early exponential cultures of MC4100(λJV304) and MCS2653 (which carries the Mu*cts62*B*am1066*pKm7701 and the λJV304 prophages) grown at 30°C in LB were induced at 42°C for either 30 or 60 min, plated on Xgal indicator plates and incubated overnight at 30°C. In this assay, blue colonies contained

Table 3. Role of Mu*cts62* prophage mutations on *araB–lacZ* fusion formation in Δ*crp*::Cm (pXJS5677*araC*^{c}) prefusion strains.

Expt.	Strain[a]	*crp*	Prophage markers	Days/32	AraLac^{+}colonies
I	RH8635	Δ[b]	WT	25	5 ± 3 (n = 8)
	RH8636	Δ	WT	25	3 ± 2 (n = 8)
	RH8635/λ	+	WT	25	335 ± 68 (n = 8)
	RH8636/λ	+	WT	25	286 ± 79 (n = 8)
II	MCS2526-31[c]	Δ	B*am1066*p*Km7701*	15	86 ± 35 (n = 4)
	MCS2526-31/λ	+	B*am1066*p*Km7701*	15	144 ± 35 (n = 4)
III	RH8630	Δ	B^{+}p*Km7701*	30	5 ± 3 (n = 8)
	RH8632	Δ	B^{+}p*Km7701*	30	6 ± 2 (n = 8)
	RH8633	Δ	B*am1066*p*Km7701*	30	24 ± 12 (n = 7)
	RH8634	Δ	B*am5179*p*Km7701*	30	77 ± 16 (n = 8)
IV	RH8630	Δ	B^{+}p*Km7701*	22	3 ± 2 (n = 4)
	RH8632	Δ	B^{+}p*Km7701*	22	5 ± 5 (n = 4)
	RH8633	Δ	B*am1066*p*Km7701*	22	55 ± 26 (n = 4)
	RH8634	Δ	B*am5179*p*Km7701*	22	74 ± 27 (n = 4)
	RH8630/λ	+	B^{+}p*Km7701*	22	243 ± 46 (n = 4)
	RH8632/λ	+	B^{+}p*Km7701*	22	275 ± 28 (n = 4)
	RH8633/λ	+	B*am1066*p*Km7701*	22	235 ± 107 (n = 4)
	RH8634/λ	+	B*am5179*p*Km7701*	22	458 ± 65 (n = 4)

a. As a control, cultures were transduced to *crp*$^{+}$ with λ(= lambda)2098 from S. Adhya.
b. The symbol Δ is a capital delta; the two alleles of crp are + and Δ (delta) = Δ*crp*::CM.
c. Four sibling transductant clones were tested.

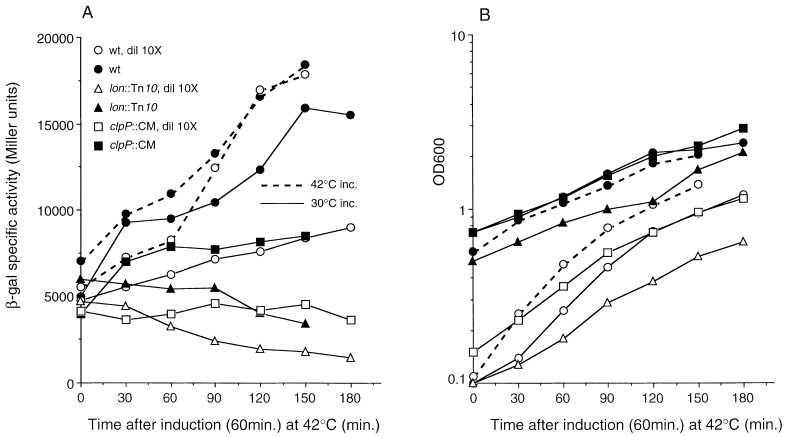

Fig. 5. Expression from pE after induction at 42°C and return to 30°C. Cultures of MC4100(λJV304) (circles) and its *clpP::*Cm (squares) and *lon::*Tn*10* (triangles) derivatives were grown to early log phase at 30°C in LB with aeration and shifted to 42°C for 60 min. At that point (time 0), the culture was diluted 10-fold in fresh LB. The concentrated and diluted cultures were aerated further at 30°C, and aliquots of the concentrated and diluted MC4100(λJV304) cultures were kept at 42°C (dotted lines). β-Galactosidase activity was measured at regular time intervals in the undiluted (closed symbols) and diluted (open symbols) cultures.
A. β-Galactosidase specific activity (Miller units) as a function of growth time (in min).
B. Growth curve; OD_{600} as a function of time.
The values are means of at least two experiments.

a majority of derepressed cells, whereas white colonies consisted of cells that were not induced or returned to the repressed state. In MC4100(λJV304), the proportion of blue colonies was higher after 60 min than after 30 min of induction. Under both conditions, they were more abundant in MCS2653, which formed exclusively blue colonies after 60 min of induction (Table 4). When the cultures were grown at 30°C for several generations after the thermal

Table 4. Analysis of thermally derepressed populations by plating on Xgal indicator medium.

Strain	Culture history before Xgal/30°C	Total colonies	Percentage of blue colonies
First experiment			
MC4100(λJV304)	42°C/30 min	529	44
	42°C/30 min, 10^{-6} in LB, ON/30°C	394	4
	42°C/60 min	215	88
	42°C/60 min, ON/30°C	228	52
	42°C/60 min, 10^{-6} in LB, ON/30°C	540	11
MCS2653 (Mu*cts62*B$^-$ Kil$^-$) (λJV304)	42°C/30 min	622	96
	42°C/30 min, 10^{-6} in LB, ON/30°C	184	82
	42°C/60 min	185	100
	42°C/60 min, ON/30°C	134	79
	42°C/60 min, 10^{-6} in LB, ON/30°C	359	89
Second experiment			
MC4100(λJV304)	42°C/60 min	578	52
	42°C/60 min, 5 h/30°C aerated	960	37
	42°C/60 min, ON/30°C aerated	1098	52
	42°C/60 min, 10^{-6} in LB, ON/30°C	2273	1.3
MC4100(λJV304) *clpP*::Cm	42°C/60 min	608	23
	42°C/60 min, 5 h/30°C aerated	935	16
	42°C/60 min, ON/30°C aerated	197	< 0.5
	42°C/60 min, 10^{-6} in LB, ON/30°C	3129	0.6
MC4100(λJV304) *lon*::Tn*10*	42°C/60 min	112	1
	42°C/60 min, 5 h/30°C aerated	319	0.3
	42°C/60 min, ON/30°C aerated	669	6
	42°C/60 min, 10^{-6} in LB, ON/30°C	1392	0.07

Cultures grown in LB with aeration to early exponential phase were induced at 42°C and shifted back to 30°C for the times indicated in the second column. Control cultures were kept at 30°C. Appropriate dilutions of heat-induced and control cultures were plated on Xgal indicator medium. No blue (derepressed) colonies were detected in the uninduced control cultures. In another control (not shown), MC4100(λJVc) grown at 30°C formed only dark blue colonies on Xgal plates incubated overnight at 30°C. All white (repressed) colonies turned light blue after 36 h incubation in the case of MC4100(λJV304), but only after 48 h or later for MCS2653 and for MC4100(λJV304) *clpP::*Cm or *lon::*Tn*10*. This probably reflects S derepression occurring during growth of the colonies on the plates. Heat-induced cultures of both MC4100(λJV304) and MCS2653 produced completely dark blue colonies after overnight incubation at 30°C, although most coloured colonies were only partially blue. All coloured colonies were scored as blue. The 10^{-6} dilutions after heat induction were grown at 30°C without aeration to avoid S derepression. The number of doublings in these cultures before plating was ≈24.

induction, the proportion of blue colonies went down, especially in the case of MC4100(λJV304). In the case of MCS2652-53, which expresses the Mu Ner protein, increasing the number of generations at 30°C by diluting the culture upon return to 30°C had no effect on the proportion of white colonies, which most probably contained cells that recovered immunity. In MC4100(λJV304), which does not express Ner, the number of white colonies went up to 90% (Table 4) in those same conditions. These results are consistent with the known property of Ner to repress pCM and hence to favour the maintenance of the derepressed state (van Leerdam *et al*., 1982). They suggested that growth at low cell density, whether on plates or in liquid medium, favoured the return to repression in cells that did not express Ner.

Observation of white and blue colonies formed upon plating of the induced *clp* and *lon* lysogens on Xgal indicator plates at 30°C confirmed the results shown in Fig. 5. The number of blue colonies was the lowest in the *lon* strain and lower in the two mutants than in the parental lysogen (Table 4). As thermal induction occurred with similar efficiency in wild-type, *clp* and *lon* strains (Table 1B and Fig. 5), ClpP and Lon appeared to retard the re-establishment of repression in at least part of the population. Twenty generations at 30°C were sufficient for most of the cells in the cultures of the parental MC4100(λJV304) and its *clpP* and *lon* derivative to return to the repressed state. Indeed, no more blue colonies could be detected in the cultures diluted to 1.0×10^3 cfu ml^{-1} and grown until reaching 1.0×10^9 cfu ml^{-1} (Table 4).

Discussion

The cts62 *mutation allows the Mu prophage to undergo a σ^s-dependent derepression at low temperature, which is under the control of the ClpXP and Lon proteases*

The formation of *araB–lacZ* coding sequence fusions was the first documented example of what is now called 'adaptive mutation' (Shapiro, 1984a; reviewed in Foster, 1993). A key question raised by this and other adaptive mutation systems is to understand the molecular basis for the dramatic effect of physiological conditions on the process of genetic change.

In this paper, we show that thermal induction triggers accelerated *araB–lacZ* fusion formation in prefusion strains that carry a mutated Mu*cts62* prophage, which cannot kill the host cell. Prophage derepression in prefusion cultures was thus an important factor in determining the kinetics of *araB–lacZ* fusion colony appearance on selective medium, as expected from the involvement of Mu transposase in *araB–lacZ* fusion formation.

The results implied that the Mu*cts62* prophage was derepressed upon prolonged incubation of lysogens at low temperature, conditions that allow for fusion formation (Shapiro, 1984a; Maenhaut-Michel and Shapiro, 1994). This was confirmed with the indicator system in which β-galactosidase expression reflects the activity of the Mu lytic promoter pE. We indeed observed significant derepression in cultures aerated at 30°C, overnight or longer, whether in minimal or rich medium. We called this process S derepression to distinguish it from thermal induction and to reflect the fact that it occurred upon starvation, in stationary cultures, was probably stochastic in the population and was σ^s dependent. S derepression most probably occurred in a fraction of the stationary phase population of Mu*cts62* lysogens, which could correspond to the fraction that was activated for fusion formation.

Among the bacterial functions that have been shown to regulate *araB–lacZ* fusion formation, ClpP, ClpX, Lon, RpoS and Crp (Shapiro, 1993; Gómez-Gómez *et al*., 1997; G. Maenhaut-Michel and J. A. Shapiro, unpublished; this paper) were tested for their effect on S derepression. All but Crp were found to be required for S derepression in the λJV304 system and, hence, were most probably necessary for fusion formation because they triggered S derepression of the Mu*cts62* prophage. Additional effects at later stages of the fusion process cannot be excluded. ClpX, for example, is a key factor in the restructuring of the strand transfer complex (STC) during Mu replicative transposition (Levchenko *et al*., 1995; Kruklitis *et al*., 1996) and could also interact with the STC intermediate during fusion formation (G. Maenhaut-Michel and J. A. Shapiro, unpublished results).

A role for ClpXP in the control of Mu repression could have been anticipated in view of the capacity of the protease to degrade Mu wild-type repressor *in vitro* (Welty *et al*., 1997). The stronger effect of Lon was more of a surprise, as previous studies provided no indication that Mu repressor is a substrate for Lon (Geuskens *et al*., 1992; J. E. Laachouch, unpublished results). The two host proteases appear to act along the same pathway, as inactivation of one of them was sufficient to reduce S derepression. A few proteins are known to be degraded by both Lon and ClpXP, the F plasmid protein CcdA, the phage λ O thermosensitive mutant protein and the umuD/D′ protein. However, Lon and ClpXP target different forms of these polypeptides. While Lon degrades wild-type CcdA (Van Melderen *et al*., 1994), UmuD homodimers (Frank *et al*., 1996) and OTs at high temperature (Gottesman and Zipser, 1978), ClpXP degrades the wild-type O protein (Gottesman *et al*., 1993; Wojtkowiak *et al*., 1993), a mutant CcdA with a modified C-terminal end (Laachouch *et al*., 1996; D. Jaloveckas, unpublished results) and UmuD/D′ heterodimers (Frank *et al*., 1996). Whether different forms of the *cts62* repressor are substrates for Lon and Clp is under investigation.

Most probably, σ^S controls Mu repression indirectly. On the one hand, σ^S does not appear to operate on the Mu pE promoter, as no difference in β-galactosidase expression could be detected at 42°C between MC4100(λJV304) and its *rpoS* derivative. On the other hand, σ^S is degraded by ClpXP during exponential growth and, hence, is stabilized in *clpX* and *clpP* strains, which express at least part of the σ^S-controlled genes in exponential growth phase as a result (Schweder *et al*., 1996). Nevertheless, we did not detect any S derepression in exponential cultures of the Clp$^-$ strains grown at 30°C. As established for σ^S expression (Huisman and Kolter, 1994), S derepression occurred upon starvation at lower cell density in diluted broth and, hence, appeared to be triggered by starvation rather than by cell density. This suggests that S derepression is related to increased expression of σ^S under starvation conditions.

*S derepression and thermal induction of Mu*cts62 *are growth phase sensitive and under different genetic controls*

Contrary to S derepression, thermal derepression was fully efficient in early exponential cultures of MC4100(λJV304) and was not affected by host *clpX*, *clpP*, *lon* and *rpoS* mutations. This is again consistent with known properties of the *cts62* repressor. Its affinity for the Mu operators is further reduced at 42°C compared with 30°C (Vogel *et al*., 1991), and repressor–operator complexes assembled at 30°C go through a drastic conformational change when they are shifted to 42°C (Vogel *et al*., 1996). Thermal induction thus most probably results from the collapse of the repressor–operator complex at 42°C rather than from repressor degradation.

In concentrated early log phase cells, in late log and stationary phase cells, thermal induction was less efficient than in early exponential phase. Thermal induction thus responded to growth phase and did so in a manner different from S derepression, as it was sensitive to cell density and was not affected by mutations that affected S derepression. The lower efficiency of thermal induction under these physiological conditions could simply reflect the general decrease in mRNA and protein synthesis occurring in stationary phase (Hengge-Aronis, 1996). It could, however, also reflect an influence of host physiology on the *cts62* repressor thermosensitivity. Indeed, that mutant repressor retains some activity at 42°C. Mu*cts62* lysogenizes at a reduced but significant frequency (Bourret and Fox, 1988), and overexpression of the *cts62* protein is known to prevent thermal induction (Zipser *et al*., 1977). In addition, as mentioned previously, repression is modulated by the host IHF, H-NS and FIS proteins, the concentration of which is influenced by growth phase (for instance, see Finkel and Johnson, 1992; Ditto *et al*., 1994; Atlung and Ingmer, 1997). Further experiments will be necessary to establish whether the growth phase effect on thermal induction results from limited activity of pE and/or decreased thermosensitivity of the *cts62* repressor in stationary phase cells.

Maintenance of the derepressed state and fate of the induced population

Testing for accelerated fusion kinetics with thermally induced Mu*cts62Bam*pKm7701 cultures indicated that the derepressed state could persist through many cell doublings at low temperature. Moreover, repressor remained undetectable by Western blotting in thermally induced Mu*cts62* lysogens returned to low temperature for several generations. The results with the reporter system in thermally induced cultures of MC4100(λJV304) also supported this view. Consistent with the fact that the Mu Ner regulator represses pCM and, hence, should stabilize the derepressed state (van Leerdam *et al*., 1982), more cells remained derepressed in strains expressing Ner. On the contrary, in *clp* and *lon* hosts, restoration of pE repression was more efficient. Besides being required for S derepression, the proteases thus also prevented the restoration of repression. The simplest interpretation of this result was, of course, that they degraded repressor, either while cells were at 42°C or once they had been returned to 30°C, or both. The temperature shift from 42°C to 30°C could, for instance, generate an abnormal configuration of Rep*cts62* that is recognized by the Clp and/or Lon proteases. Lon is known to recognize and degrade abnormally folded proteins (Gottesman, 1996). Further experiments are in progress to elucidate this question.

A role for Crp in araB–lacZ *fusion formation*

Our results showed that, in MuKil$^+$ or Kil$^-$ prefusion strains, Crp was required for *araB–lacZ* fusion formation when MuB was present. In the absence of MuB, a partial bypass of this requirement was observed. Removal of *B*, *kil* and the other open reading frames (ORFs) in the pKm7701 substitution did not inhibit *araB–lacZ* fusion formation in a Crp$^+$ background, which was consistent with the fact that the MuB protein is not essential for transposition and other Mu-induced rearrangements (deletions, inversions, replicon fusions; Faelen *et al*., 1978). We also found that conditions that favoured S derepression accelerated the appearance of fusion colonies in MuB$^-$Kil$^-$ prefusion strains but decreased the yield of fusion colonies when the prophage was B$^+$Kil$^+$. Expression of MuB and Kil proteins thus appeared to prevent fusion formation. The negative effect of MuB is consistent with known properties of the protein, which should be expressed along with MuA transposase, Kil and other early proteins upon S derepression

of a Mu*cts62* lysogen. Expression of MuA (in physiological quantity) is not lethal for the host (Faelen *et al*., 1979). However, coincident expression of MuA and MuB or the sole expression of the Kil protein are each sufficient to kill the host (van de Putte *et al*., 1977; Waggoner *et al*., 1984). MuB is known to stimulate all the steps leading to MuA-promoted STC formation *in vitro* (Naigamwalla and Chaconas, 1997) and to inhibit the STC disassembly and the commitment to replication by controlling the access of ClpX to the transposase (Levchenko *et al*., 1997). A very small amount of STC has been detected in MuB$^-$ lysogens *in vivo* (Pato *et al*., 1995). In a MuB$^+$Kil$^+$ (or Kil$^-$) lysogen, derepression should lead to multiple B-stimulated rounds of replication and thus provoke cell death and prevent fusion formation.

The negative role of MuB on fusion formation could also be assigned to MuB-dependent transposition immunity that targets transposition to sites distant from the donor Mu termini (Maxwell *et al*., 1987; Adzuma and Mizuuchi, 1989; 1991; Manna and Higgins, 1999).

In stationary phase cultures of Crp$^+$ MuB$^+$Kil$^+$ lysogens, at least a fraction of the S-derepressed population remains available to generate rearranged progeny with *araB–lacZ* fusions. It is thus tempting to hypothesize that these cells become phenotypically B$^-$Kil$^-$ so that they survive S derepression. The fact that Crp was essential for fusion formation when MuB was present suggests that Crp or some Crp-dependent function(s) could antagonize B-dependent cell death and thus allow for fusion formation. B-dependent transposition immunity could also be bypassed, allowing for targeting strand transfer very close to the resident Mu*cts62* prophage, where it has to occur to generate *araB–lacZ* fusions (Fig. 1D; Maenhaut-Michel and Shapiro, 1994; Maenhaut-Michel *et al*., 1997). Thus, antagonism of MuB function provides two possibilities for Crp-dependent activation of *araB–lacZ* fusion formation under starvation conditions.

Besides S derepression of the Mu*cts62* prophage and the second step that was controlled by Crp, the requirement of at least one additional starvation-controlled function(s) was indicated by the fact that starvation was still necessary for fusion completion in thermally induced B$^-$Kil$^-$ lysogens when the Crp function was not essential.

Biological significance

Our results show that the R47Q mutation in the Mu*cts62* repressor protein integrates Mu repression and derepression into the host σ^S regulon. This provides the host cell with a new potential for adaptation under starvation conditions, as it allows it to profit from the Mu transposition machinery to perform an 'adaptive' rearrangement. Whether the wild-type repressor also participates in this control circuit remains an open question. S derepression did not occur in bacteria in which pE was repressed by either the wild-type or the *cts,sts* repressor. Compared with *cts62*, these two proteins bind Mu operators with a higher affinity and, for the wild type, with lower co-operativity (Vogel *et al*., 1991; 1996). Weaker repressor–operator interactions resulting from the *cts62* R47Q mutation would then allow the Mu lytic/lysogeny regulation to become part of the host σ^S-dependent regulatory cascade. S derepression was probably triggered by a decrease in the concentration of active repressor and/or host proteins involved in Mu repression/derepression below the threshold amount required for efficient repression by the *cts62* repressor at low temperature. Likewise, this amount of repressor would be sufficient to ensure stable repression by the more efficient wild-type and *cts,sts* proteins. More careful analysis of 'spontaneous' Mu c^+ derepression during the growth cycle and in various host genetic backgrounds should shed light on this problem. Until then, the applicability of S derepression to wild-type Mu prophage biology remains uncertain.

Nonetheless, in addition to the specific growth phase effects on Mu*cts62* repression and derepression, our results illustrate that the study of *araB–lacZ* fusions provides insights into regulatory connections between Mu biology and *E. coli* control circuits. As for other temperate bacteriophages such as λ, additional layers of regulatory interactions between phage Mu and its host become apparent. It is logical to assume that these regulatory interactions evolved to enhance the mutual benefits that phage and host can derive from Mu's dual lifestyle as an autonomous virus and a cellular transposable element.

Experimental procedures

Strains

Bacteria, phages and plasmids used in this study are listed in Table 5. Most bacterial strains derive from MC4100. It was lysogenized with, λJV300, λJV304 or λJV313 by selecting for Km^r. The *clpP*::Cm, *clpXam14*, *lon::*Tn*10*, *rpoS*::Tn*10* and *crp*::Cm mutations were transduced by P1 in the MC4100(λJV304). MCS2652 and MCS2653 carry the Mu*cts62Bam1066*pKm7701 prophage and the λJV304. They were derived from MCS1965 (see below) by UV curing of its λp1(209) prophage and then lysogenized with λJV304 by selecting for λ immunity and testing for resistance to higher kanamycin concentrations (200 or 400 μg ml^{-1}).

Figure 1A shows the structure of the B$^-$ Kil$^-$ prophage Mu*cts62Bam*pKm7701 in a series of prefusion strains (Fig. 1D) carrying one of four different *Bam* mutations (*am1066*, *am5176*, *am5179* and *am7154*; O′Day *et al*., 1979). In pKm7701 prophages, a fragment of Tn*5* substitutes for a segment of the Mu early region (4.4–7.2 kb from the Mu left end; Ross *et al*., 1986). The pKm7701 substitution has not been sequenced. Based on end-point determinations, the deletion of Mu material removes the *kil* and *gam* sequences

Table 5. Bacterial strains, phages and plasmids.

Bacteria		
MC4100	*araD139*,D(*lacIPOZYA,argF*)U169, *fla,relA,rpsL*	Casadaban (1976)
MC4100*clpXam14*	Same as above but *clpXam14*	L. Desmet
MC4100*clpP*::Cm	Same as above but *clpP*::Cm, P1 transduction of *clpP*::Cm from SG22030 into MC4100	Mahmmedi-Alaoui *et al*. (1994)
MCS2	As MC4100 but *ara*::+Mu*cts62*(λp1(209))	Shapiro (1984a)
MCS216a2 and 2b31	Two subclones derived from MCS2	Maenhaut-Michel and Shapiro (1994)
MCS1965	As MCS2 but Mu*cts62Bam1066*::pKm7701	Maenhaut-Michel *et al*. (1997)
MCS1969	As MCS2 but Mu*cts62Bam5176*::pKm7701	
MCS1972	As MCS2 but Mu*cts62Bam5179*::pKm7701	
MCS1977	As MCS2 but Mu*cts62Bam7154*::pKm7701	
MCS2652-53	Same as MCS1965 but cured from λp1(209) and lysogenized with λJV304	This work
Phages		
λRS45		Simons *et al*. (1987)
λJV300	λRS45 with the left end of Muc^+ fused to *lacZ*	Vogel *et al*. (1991)
λJV304	As above but with Mu*cts62* left end	Vogel *et al*. (1991)
λJV313	As above but Mu*cts62*,*sts*62-1 left end	Vogel *et al*. (1991)
λJVc	λJV300 with a *c* mutation	A. Toussaint
Mu*cts62*pAp1	Mu*cts62* with an Ap^r marker	Leach and Symonds (1979)
Mu18-1	Mu*cts62*A^-B^-pAp1	Résibois *et al*. (1981)
Plasmids		
pGRAL1	pBluescript-SK+ with left end *Hin*dIII fragment of Mu*cts62*	This work

as well as ORFs for the putative E5–E13 and F-86 polypeptides (Priess *et al*., 1987). All B^-Kil^- prefusion strains showed 100% survival at 42°C on complex medium, independently of *Bam* allele (MCS1965 – *Bam1066*; MCS1969 – *Bam5176*; MCS1972 – Bam*5179*; MCS1977 – *Bam7154*).

Construction of Δcrp::Cm, $araC^c$ *prefusion strains*

An *araC*^{c} mutation was isolated on plasmid pXJS5672 carrying the entire *ara* operon in a Crp^+ host by selecting colonies on arabinose minimal medium in the inhibition zone surrounding a filter paper disk impregnated with L-fucose solution (Englesberg *et al*., 1965). A 1.4 kb *Bam*H1 *araC*^{c} fragment from pXJS5672 was subcloned into pBR322, and the resulting pXJS5677 plasmid transformed *crp*::Cm strains to Ara^+ (Heffernan *et al*., 1976). The pXJS5677 plasmid also activated β-galactosidase synthesis in a Δ*crp*::Cm strain carrying an *araB–lacZ* fusion; the activity was arabinose inducible from a high basal level. The Δ*crp*::Cm mutation and the pXJS5677 plasmid were then introduced in MCS2 and its B^+p*Km7701* and B^-p*Km7701* derivatives by sequential P1 transduction (selecting for Cm^r) and transformation (selecting for Ap^r).

Media

Bacteria were grown in LB (Lennox, 1955) or minimal liquid 132 medium (MM) (Glansdorff, 1965) supplemented with glucose as mentioned (MM Glu). MM/10%LB is a 10-fold dilution of LB in minimal 132 medium at pH 7.2 without a carbon source, and H2O/10%LB is a 10-fold dilution of LB in water. Solid media (LA and minimal) were the same but supplemented with 1.2% Difco agar. Minimal AraLac-selective medium contained 0.2% arabinose and 0.2% lactose as described previously (Shapiro, 1984a; Maenhaut-Michel and Shapiro, 1994). Xgal indicator agar was TA12 tryptone agar (Kaiser, 1955) supplemented with 40 μg ml^{-1} Xgal. Kanamycin (Km; 25 μg ml^{-1}), ampicillin (Ap; 20 μg ml^{-1} for the selection of MupAp1 lysogens, 50 μg ml^{-1} for the selection of plasmids) and chloramphenicol (Cm; 25 μg ml^{-1}) were included where appropriate.

Microbiological and genetic methods

The classical protocols for phage manipulations were performed as described previously (Arber *et al*., 1983 for λ; Bukhari and Ljundquist, 1977 for Mu; Lennox, 1955 for P1 transduction). Assaying for *araB–lacZ* fusion formation was carried out as described previously (Shapiro, 1984a; Maenhaut-Michel and Shapiro, 1994).

Replica plate testing for derepression and fusion formation was performed as follows. Single colonies from 32°C LA plates were tested for their ability to produce fusions by patching them on LA, incubating for > 8 h at 32°C and replica plating the thick patches to AraLac-selective medium containing 0.01% glucose and 30 μg ml^{-1} Xgal. The glucose permitted limited growth of the replica patches on selective agar and accelerated fusion formation, as observed previously (Shapiro, 1984a). The patches were made either by repeated stabbing directly from a single colony into a small zone of the master plate (Fig. 3A) or by resuspending a single colony in liquid medium, growing the single colony culture in static culture at room temperature to $1–2 \times 10^9$ cfu ml^{-1} and spotting 10 μl on the master plate. The replica plates were incubated at 32°C and scored daily for the appearance of dark blue fusion colonies.

Time course of β-galactosidase expression

Overnight cultures were grown in LB overnight at 30°C without aeration (except when indicated) from isolated colonies

on LA or a −80°C frozen stock culture in 50% glycerol. After a 100-fold dilution in fresh LB (or MM Glu 0.4%), the cultures were grown with aeration at the temperature indicated. They were sampled at various times for titration (on LA at 30°C), OD and β-galactosidase measurements. Thermal induction was for 30 or 60 min (as specified in Tables and Figures) at 42°C with aeration. β-Galactosidase specific activity was measured and calculated according to the following formula (Miller, 1972):

$$\text{specific activity} = 1000 \times [OD_{420} - 1.75 \times OD_{550}]/[t \times v \times OD_{600}],$$

where t is the length of the assay in min and v is the volume of the culture used in the assay, in ml.

PCR analysis

PCR amplification was performed using the primers *ara7* and *lac6* described previously (Maenhaut-Michel and Shapiro, 1994). Aliquots of the cultures (between 1 and 10 μl) were tested directly in the assay. It consisted of 5 μl of PCR buffer diluted 10 times with $MgCl_2$ (1.5 mM final concentration) from Boehringer Mannheim, 1 μl dNTP mix (0.2 mM of each, final concentration) from Finnzymes, 1 μM each primer, 1 U (1 μl) of *Taq* polymerase (Boehringer Mannheim) in a total volume of 50 μl adjusted with water. The amplification was performed in a Perkin-Elmer 'Geneamp' PCR system 9600 with the following programme: 5 min at 94°C followed by 35 cycles of a three-temperature programme (1 min at 94°C, 1 min at 55°C, 1 min at 72°C plus a 5 s extension), 10 min at 72°C and hold at 4°C.

Western blotting for Rep62

Independent single-colony cultures of the Mu*cts62-Bam5174*pKm7701 prefusion strain MCS1977 were grown in LB at 32°C to a density of $\approx 1 \times 10^8$ cfu ml^{-1}. One aliquot of each culture was thermally induced (60 min incubation at 42°C with gentle aeration), while another aliquot was maintained at 32°C. After induction, the cultures were diluted 20-fold in fresh LB and incubated overnight at 32°C. Samples were taken from 32°C cultures at 6–7 × 10^9 cfu ml^{-1} (control) and from induced cultures after dilution and overnight growth at 32°C at 10^{10} cfu ml^{-1} (test sample), sampling 1 ml portions and storing the pellets frozen at −20°C.

Western blot analysis was conducted as described previously (Sambrook *et al*., 1989), using polyclonal rabbit antibodies (a kind gift from Dr N. Patrick Higgins, University of Alabama at Birmingham) raised against purified Rep and using the enhanced chemiluminescence detection system purchased from Amersham. Cellular proteins were resolved on a 15% SDS–polyacrylamide gel before blotting onto a nitrocellulose membrane, and sampled cells were prepared for electrophoresis as follows. To 4×10^9 cfu suspended in 20 μl [10 mM Tris-HCl, pH 7.5, 10% (w/v) glycerol], lysozyme was added to a final concentration of 200 μg ml^{-1}, and the mixture was placed on ice for 45 min. Lysis was completed by five cycles of freezing in liquid nitrogen and thawing in ice water. The solution was adjusted to 10 mM $MgCl_2$, and DNase I and RNase A were added to a final concentration of 50 μg ml^{-1} each. The mixture was placed on ice for an additional 60 min and heated at 37°C for 2 min, and then EDTA was added to a final concentration of 12 mM. An 8 μl portion of this extract (equivalent to 1×10^9 cfu) was mixed with SDS loading buffer [50 mM Tris-HCl, pH 7.5, 10% (w/v) glycerol, 1% SDS, bromophenol blue] and heated to 100°C for 5 min before electrophoresis.

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References

Adzuma, K., and Mizuuchi, K. (1989) Interaction of proteins located at a distance along DNA: mechanism of target immunity in the Mu DNA strand-transfer reaction. *Cell* **57:** 41–47.

Adzuma, K., and Mizuuchi, K. (1991) Steady-state kinetic analysis of ATP hydrolysis by the B protein of bacteriophage Mu. Involvement of protein oligomerization in the ATPase cycle. *J Biol Chem* **266:** 6159–6167.

Alazard, R., Betermier, M., and Chandler, M. (1992) *Escherichia coli* integration host factor stabilizes bacteriophage Mu repressor interactions with operator DNA *in vitro*. *Mol Microbiol* **6:** 1707–1714.

Arber, W., Enquist, L., Hohn, B., Murray, N., and Murray, K. (1983) Experimental methods for use with lambda. In *LambdaII*. Hendrix, R.W., Roberts, J.W., Stahl, F.W., and Weisberg, R.A. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 433–466.

Atlung, T., and Ingmer, H. (1997) H-NS: a modulator of environmentally regulated gene expression. *Mol Microbiol* **24:** 7–17.

Bétermier, M., Poquet, I., Alazard, R., and Chandler, M. (1993) Involvement of *Escherichia coli* FIS protein in maintenance of bacteriophage Mu lysogeny by the repressor: control of early transcription and inhibition of transposition. *J Bacteriol* **175:** 3798–3811.

Bétermier, M., Rousseau, P., Alazard, R., and Chandler, M. (1995) Mutual stabilisation of bacteriophage Mu repressor and histone-like proteins in a nucleoprotein structure. *J Mol Biol* **249:** 332–341.

Bourret, R.B., and Fox, M.S. (1988B) Lysogenization of *Escherichia coli* Him+, *himA*, and *himD* hosts by bacteriophage Mu. *J Bacteriol* **170:** 1672–1682.

Bukhari, A.I., and Ljungquist, E. (1977) Bacteriophage Mu: methods for cultivation and use. In *DNA Insertion Elements, Plasmids and Episomes*. Bukhari, A.I., Shapiro J.A., and Adhya, S.L. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 749–756.

Casadaban, M. (1976) Transposition and fusion of the lac gene to selected promoters in *E. coli* using bacteriophage lambda and Mu. *J Mol Biol* **104:** 541–555.

Ditto, M.D.M., Roberts, D., and Weisberg, R.A. (1994) Growth phase variation of integration host factor level in *Escherichia coli*. *J Bacteriol* **176:** 3738–3748.

van Drunen, C.M., van Zuylen, C., Mientjes, E.J., Goosen, N., and van de Putte, P. (1993) Inhibition of bacteriophage Mu transposition by Mu repressor and Fis. *Mol Microbiol* **10:** 293–298.

Englesberg, E., Irr, J., Power, J., and Lee, N. (1965) Positive control of enzyme synthesis by gene C in the L-arabinose system. *J Bacteriol* **90:** 946–957.

Faelen, M., Huisman, O., and Toussaint, A. (1978) Involvement of phage Mu-I early functions in Mu-mediated chromosomal rearrangements. *Nature* **271:** 580–582.

Faelen, M., Résibois, A., and Toussaint, A. (1979) Mini-Mu. An insertion element derived from temperate phage Mu-1. *Cold Spring Harbor Symp Quant Biol* **43:** 1169–1177.

Falconi, M., Odle, J., Hillyard, D., Gualerzi, C., and Higgins, N.P. (1991) Mutations altering chromosomal protein H-NS activate phage Mu transposition. *New Biologist* **3:** 615–625.

Finkel, S.E., and Johnson, R.C. (1992) The Fis protein: it's not just for DNA inversion anymore. *Mol Microbiol* **6:** 3257–3260.

Foster, P.L. (1993) Adaptive mutation: the uses of adversity. *Annu Rev Microbiol* **47:** 467–504.

Frank, E.G., Ennis, D.G., Gonzales, M., Levine, A.S., and Woodgate, R. (1996) Regulation of SOS mutagenesis by proteolysis. *Proc Natl Acad Sci USA* **93:** 10291–10296.

Gama, M.J., Toussaint, A., and Higgins, N.P. (1992) Stabilization of bacteriophage Mu repressor-operator complexes by the *Escherichia coli* integration host factor protein. *Mol Microbiol* **6:** 1715–1722.

Geuskens, V., Vogel, J.L., Grimaud, R., Desmet, L., Higgins, N.P., and Toussaint, A. (1991) Frameshift mutations in bacteriophage Mu repressor can confer a *trans*-dominant virulent phenotype to the phage. *J Bacteriol* **173:** 6578–6585.

Geuskens, V., Mhammedi-Alaoui, A., Desmet, L., and Toussaint, A. (1992) Virulence in bacteriophage Mu: a case of *trans*-dominant proteolysis by the *E. coli Clp* serine-protease. *EMBO J* **11:** 5121–5127.

Glansdorff, N. (1965) Topography of cotransducible arginine mutations in *E. coli* K12. *Genetics* **51:** 167–179.

Gómez-Gómez, J.M., Blásquez, J., Baquero, F., and Martínez, J.L. (1997) H-NS and RpoS regulate emergence of Lac Ara+ mutants of *Escherichia coli* MCS2. *J Bacteriol* **179:** 4620–4622.

Goosen, N., and van de Putte, P. (1987) Regulation of transcription. In *Phage Mu*. Symonds, N., Toussaint, A., van de Putte, P., and Howe, M.M. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 41–52.

Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annu Rev Genet* **30:** 465–506.

Gottesman, S., and Zipser, D. (1978) Deg phenotype of *Escherichia coli lon* mutants. *J Bacteriol* **133:** 844–851.

Gottesman, S., Clark, W.P., de Crecy-Lagard, V., and Maurizi, M.R. (1993) ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*. *J Biol Chem* **268:** 22618–22626.

Heffernan, L., Bass, R., and Englesberg, E. (1976) Mutations affecting catabolite repression of the L-arabinose regulon in *Escherichia coli* B/r. *J Bacteriol* **126:** 1119–1131.

Hengge-Aronis, R. (1996) Regulation of gene expression during entry into stationary phase. In Escherichia coli *and* Salmonella*: Cellular and Molecular Biology*, 2nd edn. Neidhardt, F.C., *et al*. (eds). Washington, DC: American Society for Microbiology Press, pp. 1497–1512.

Howe, M.M. (1973) Prophage deletion mapping of bacteriophage Mu-1. *Virology* **54:** 93–101.

Huisman, G.W., and Kolter, R. (1994) Sensing starvation: a homoserine lactone-dependent signaling pathway in *Escherichia coli*. *Science* **265:** 537–539.

Kaiser, A.D. (1955) A genetic study of the temperate coliphage. *Virology* **1:** 424–443.

Krause, H.M., and Higgins, N.P. (1986) Positive and negative regulation of the Mu operator by Mu repressor and *E. coli* integration host factor. *J Biol Chem* **261:** 3744–3752.

Kruklitis, R., Welty, D.J., and Nakai, H. (1996) ClpX protein of *Escherichia coli* activates bacteriophage Mu transposase in the strand transfer complex for initiation of Mu DNA synthesis. *EMBO J* **15:** 935–944.

Laachouch, J., Desmet, L., Geuskens, V., Grimaud, R., and Toussaint, A. (1996) Bacteriophage Mu repressor as a target for the *Escherichia coli* ATP-dependent Clp protease. *EMBO J* **15:** 437–444.

Leach, D., and Symonds, N. (1979) The isolation and characterization of a plaque-forming derivative of bacteriophage Mu carrying a fragment of Tn*3* conferring ampicillin resistance. *Mol Gen Genet* **172:** 179–184.

Leathers, T.D., Noti, J., and Umbarger, H.E. (1979) Physical characterization of *ilv–lac* fusions. *J Bacteriol* **140:** 251–260.

van Leerdam, E., Karreman, C., and van de Putte, P. (1982) Ner, a Cro-like function of bacteriophage Mu. *Virology* **123:** 19–28.

Lennox, E.S. (1955) Transduction of linked genetic characters of the host bacteriophage P1. *Virology* **1:** 190–206.

Levchenko, I., Luo, L., and Baker, T. (1995) Disassembly of the Mu transposase tetramer by the ClpX chaperone. *Genes Dev* **9:** 2399–2409.

Levchenko, I., Yamauchi, M., and Baker, T. (1997) ClpX and MuB interact with overlapping regions of Mu transposase: implications for control of the transposition pathway. *Genes Dev* **11:** 1561–1572.

Maenhaut-Michel, G., and Shapiro, J.A. (1994) The roles of starvation and selective substrates in the emergence of *araB–lacZ* fusion clones. *EMBO J* **13:** 5229–5239.

Maenhaut-Michel, G., Blake, C.E., Leach, D.R., and Shapiro, J.A. (1997) Different structures of selected and unselected *araB–lacZ* fusions. *Mol Microbiol* **23:** 1133–1145.

Manna, D., and Higgins, N.P. (1999) Phage Mu transposition

immunity reflects supercoil domain structure of the chromosome. *Mol Microbiol* (in press).
Maxwell, A., Craigie, R., and Mizuuchi, K. (1987) B protein of bacteriophage Mu is an ATPase that preferentially stimulates intermolecular DNA strand transfer. *Proc Natl Acad Sci USA* **84:** 699–703.
Mhammedi-Alaoui, A., Pato, M., Gama, M.J., and Toussaint, A. (1994) A new component of bacteriophage Mu replicative transposition machinery: the *E.coli* ClpX protein. *Mol Microbiol* **11:** 1109–1116.
Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Mittler, J., and Lenski, R.E. (1990) Further experiments on excisions of Mu from *Escherichia coli* MCS2 cast doubt on directed mutation hypothesis. *Nature* **344:** 173–175.
Naigamwalla, D.Z., and Chaconas, G. (1997) A new set of transposition intermediates: alternate pathways of target capture preceding strand transfer. *EMBO J* **16:** 5227–5234.
O'Day, K., Schultz, D., Ericsen, W., Reawluk, L., and Howe, M. (1979) Correction and refinement of the genetic map of bacteriophage Mu. *Virology* **93:** 320–328.
Pato, M.L., Karlok, M., Wall, C., and Higgins, N.P. (1995) Characterization of Mu prophage lacking the central strong gyrase binding site: localization of the block in replication. *J Bacteriol* **177:** 5937–5942.
Priess, H., Bräuer, B., Schmidt, C., and Kamp, D. (1987) Sequence of the left end of Mu. In *Phage Mu*. Symonds, N., Toussaint, A. van de Putte, P., and Howe, M. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 277–296.
Résibois, A., Toussaint, A., van Gijsegem, F., and Faelen, M. (1981) Physical characterization of mini-Mu and mini-D108. *Gene* **14:** 103–113.
van Rijn, P.A., Goosen, N., and van de Putte, P. (1988) Integration host factor of *Escherichia coli* regulates early and repressor transcription of bacteriophage Mu by two different mechanisms. *Nucleic Acids Res* **16:** 4595–45605.
van Rijn, P.A., Goosen, N., Turk, S.C.H., and van de Putte, P. (1989) Regulation of Mu repressor transcription by IHF depends on the level of the early transcription. *Nucleic Acids Res* **17:** 10203–10211.
Ross, W., Shore, S.H., and Howe, M. (1986) Mutants of *Escherichia coli* defective for replicative transposition of bacteriophage Mu. *J Bacteriol* **167:** 905–919.
Saier, Jr, M.H., Ramseier, T.M., Reizer, J. (1996) Regulation of carbon utilization. In Escherichia coli *and* Salmonella*: Cellular and Molecular Biology*, 2nd edn. Neidhardt, F.C., *et al*. (eds). Washington, DC: American Society for Microbiology Press, pp. 1325–1343.
Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 1847–1873.
Schweder, T., Lee, K.H., Lomovskaya, O., and Matin, A. (1996) Regulation of *Escherichia coli* starvation sigma factor (sigma s) by ClpXP protease. *J Bacteriol* **178:** 470–476.
Shapiro, J.A. (1984a) Observations on the formation of clones containing *araB–lacZ* cistron fusions. *Mol Gen Genet* **194:** 79–90.
Shapiro, J.A. (1984b) The use of Mu*dlac* transposons as tools for vital staining to visualize clonal and non-clonal patterns of organization in bacterial growth an agar and non-agar surface. *J Gen Microbiol* **130:** 1169–1181.
Shapiro, J.A. (1993) A role for the Clp protease in activating Mu-mediated DNA rearrangements. *J Bacteriol* **175:** 2625–2631.
Shapiro, J.A., and Higgins, N.P. (1989) Differential activity of a transposable element in *Escherichia coli* colonies. *J Bacteriol* **171:** 5975–5986.
Shapiro, J.A., and Leach, D. (1990) Action of a transposable element in coding sequence fusions. *Genetics* **126:** 293–299.
Simons, R.W., Houman, F., and Kleckner, N. (1987) Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53:** 85–96.
Symonds, N., Toussaint, A., van de Putte, P., and Howe, M.M. (1987) *Phage Mu*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Toussaint, A., and Résibois, A. (1983) Phage Mu: Transposition as a life-style. In *Mobile Genetic Elements*. Shapiro, J.A. (ed.). New York: Academic Press, pp. 105–158.
Toussaint, A., Desmet, L., Faelen, M., Alazard, R., Chandler, M., and Pato, M. (1987) *In vivo* mutagenesis of bacteriophage Mu transposase. *J Bacteriol* **169:** 5700–5707.
van Ulsen, P., Hillebrand, M., Zulianello, L., van de Putte, P., and Goosen, N. (1996) Integration host factor alleviates the H-NS-mediated repression of the early promoter of bacteriophage Mu. *Mol Microbiol* **21:** 567–578.
van de Putte, P., Westmaas, G.C., and Wijffelman, C. (1977) On the *kil* gene of bacteriophage Mu. In *DNA Insertion Elements, Plasmids and Episomes*. Bukhari, A.I., Shapiro, J.A., and Adhya, S.L. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 287–294.
Van Melderen, L., Bernard, P., and Couturier, M. (1994) Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. *Mol Microbiol* **11:** 1151–1157.
van Vliet, F., Couturier, M., Desmet, L., Faelen, M., and Toussaint, A. (1978) Virulent mutants of temperate phage Mu-1. *Mol Gen Genet* **160:** 195–202.
Vogel, J.L., Zhu Juan, L., Howe, M.M., Toussaint, A., and Higgins, N.P. (1991) Temperature sensitive mutations in bacteriophage Mu *c* repressor define a DNA binding site. *J Bacteriol* **173:** 6568–6577.
Vogel, J.L., Geuskens, V., Desmet, L., Higgins, N.P., and Toussaint, A. (1996) C-terminal deletions can suppress temperature-sensitive mutations and change dominance in the phage Mu repressor. *Genetics* **142:** 661–672.
Waggoner, B.T., Marrs, C.F., Howe, M.M., and Pato, M.L. (1984) Multiple factors and processes involved in host cell killing by bacteriophage Mu: characterization and mapping. *Virology* **136:** 168–185.
Welty, D.J., Jones, J.M., and Nakai, H. (1997) Communication of ClpXP protease hypersensitivity to bacteriophage Mu repressor isoforms. *J Mol Biol* **272:** 31–41.
Wojtkowiak, D., Georgopoulos, C., and Zylicz, M. (1993) Isolation and characterization of ClpX, a new ATP-dependent specificity component of the Clp protease of *Escherichia coli*. *J Biol Chem* **268:** 22609–22617.
Zipser, D., Moses, P., Kahmann, R., and Kamp, D. (1977) The molecular cloning of the immunity gene of phage Mu. *Gene* **2:** 263–271.